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EFFECT OF PARATHYROID EXTRACT AND CALCIFEROL ON THE TISSUES OF THE NEPHRECTOMIZED RAT *

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Albright and Ellsworth [1] and Ellsworth [2] have developed the idea that the activity of the parathyroid glands is dependent on the excretory function of the kidney. Since increased phosphate excretion quickly follows administration of parathyroid extract they suggested that the outpouring of phosphorus into the urine is a primary hormone effect, which is followed by a resultant rise in serum calcium. Recently Ellsworth and Futcher [3] have reported that they have obtained a rise in the serum calcium of nephrectomized dogs after the injection of parathyroid extract. They have concluded that the elimination of phosphorus in increased amount by the kidneys is not necessary in the dog for the elevation in the serum calcium that occurs after the injection of a suitable dose of parathyroid extract.

Three years ago two of the authors [4, 5, 6] began an experimental study of calcium and phosphorus metabolism and of hormone action in nephrectomized rats and dogs. Our data indicate that the presence of functional kidney tissue is a requisite for the elevation of blood calcium that characterizes overdosage with parathyroid extract. While our experiments were in progress Collip and coworkers [7] reported an examination of the bones of 8 nephrectomized rats that had been injected with parathyroid extract. On the basis

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of the osteoclastic resorption observed they concluded, without chemical data, that hormone action was independent of kidney function and interpreted the evidence as a refutation of the hypothesis first developed by Albright and Ellsworth.[1, 2] The present paper, in which tissue effects are described, is an effort to clarify and correlate some of the histological and chemical changes brought about by parathyroid hormone and vitamin D.

Since parathyroid bone lesions were described by Jaffé, Bodansky and Blair [8] numerous articles dealing with the skeletal changes that follow overdosage with both parathyroid hormone and vitamin D have appeared. Most workers agree that the bone lesions produced by these two substances are in certain respects dissimilar. Shelling,[9] using rats which weighed 50 gm., found that a chronic overdosage with viosterol gave decalcification without the fibrous replacement produced by parathyroid extract. Selye [10] likewise found fibrosis to be a prominent feature of chronic parathyroid hormone treatment. We were especially concerned with the production of acute lesions, and in acute experiments on 14 day old rats Selye observed a disappearance of bone trabeculae and the presence of osteoclasts as early as 20 hours after a dose of 10 units of parathyroid extract. Ham and Lewis [11] regard the osteoclast reaction as a change secondary to bone softening and state: "As a matter of fact the theory which postulates parathyroid hormone to act through osteoclasts is founded on a hypothesis which has been, for the most part, uncritically accepted for so many years that it has gained all the prestige of a fact." In our experiments with nephrectomized rats we have been much impressed with the value of the osteoclast reaction as an indicator of bone resorption.

Methods and Materials

The Wistar Institute albino rat was used. The experiments were acute, most of them terminating after 48 hours. Blood for the chemical analyses was withdrawn from the heart of the living rat without anesthesia. The calcium determinations were made by the Kramer-Tisdall method as modified by Tweedy and Koch.[12] In all instances calcium analyses were made on individual samples, consisting usually of 2 cc. of serum, but in a few instances of 1 cc.

Histological Procedure

The femurs, fixed in 10 per cent formalin after decalcification for a minimum time in dilute nitric acid, were embedded in paraffin and the lower end of the femur sectioned in a plane to include the full curvature of the lower end of that bone. The descriptions and interpretations were made from an examination of the central portion of the metaphysis directly beneath the cartilage plate. In this location in the rats of the age used there are relatively few osteoclasts. This cell structure is readily and accurately identified and recognition of bone resorption was not attempted except in connection with osteoclast reaction. The osteoblastic component varies much with age and, since our examination was chiefly concerned with bone resorption, the osteoblastic variations have not been emphasized. After hormone administration spindle shaped cells (Fig. 1) appear and lend a peculiar appearance to the tissue. At certain stages of functional development of osteoblasts the structural differentiation of osteoblasts and fibroblasts is not easy and we have not attempted it.

Owing to the frequency with which lesions are produced by overdosage with parathyroid extract in the stomach (dog) and the heart (dog and rat), microscopic sections of the stomach were examined in many of the rats, and sections of the myocardiums of all animals were examined. Destructive lesions, necrosis, or hemorrhage with necrosis, were not found. In some animals necrotic or fibrotic foci were found in the heart muscle of rats that had been subjected to a prior heart puncture.

Action of Parathyroid Hormone on the Bilaterally Nephrectomized Rat

Following operation the animals were not fed but were supplied with drinking water. It was found that fasting during a 48 hour period affected neither the calcium level nor the bone of the normal rat (Rats 1–3, Table I). In 9 rats that were bilaterally nephrectomized the serum calcium was within normal limits at the end of 48 hours, and the bone, on microscopic examination, was found to be normal (Rats 4–12, Table I). The remaining nephrectomized rats (Table I) were injected with parathyroid extract,* and all showed

* The parathyroid extract was generously supplied by Eli Lilly and Company.

characteristic bone changes (Fig. 1). In 5 out of 11 rats (Rats 13–23, Table I) the resorption was so extreme that spontaneous separation in the zone beneath the cartilage plate occurred during removal of the bone. The softening was accompanied by the increased osteoclastic reactions described by Collip and co-workers.[7] On the other hand the calcium, which averaged 12.36 mg. per cent in 4 of the animals, was not much changed.

TABLE I

Effect of Parathyroid Extract on the Serum Calcium and on the Femurs of Bilaterally Nephrectomized Rats *

Rat No.	Weight (individual or average)	Units parathyroid extract injected	Hrs. after sacrifice	Serum calcium	Femur
	gm.			*mg./%*	
1–3	89		48	12.06	Normal
4–12	151		48	10.94	Normal
13	107	250 x 4	48	13.64	
14	98	250 x 4	48	12.71	oc † + + + ob (spindle)
15	114	250 x 4	46		oc + + + ob (spindle)
16	111	250 x 3	26		oc + + + ob (spindle)
17	107	250 x 4	48		oc + + + ob (spindle)
18	127	250 x 4	48	10.48	oc + + + ob (spindle)
19	137	500 x 3	48	12.64	oc + + + ob (spindle)
20	107	250 x 3	26		oc + + + ob (spindle)
21	90	250 x 3	26		oc + + + ob (spindle)
22	90	250 x 3	26		oc + + + ob (spindle)
23	122	250 x 3	26		oc + + + ob (spindle)

* Rats 1–3 were normal animals that were not fed for 48 hours. Rats 4–12 were nephrectomized, but not injected.
The potency of the parathyroid extract is expressed in "Hanson" units, one of which is one-fifth of a parathormone (Collip) unit.
† oc = osteoclasts with the degree of resorption and associated osteoclastic reaction indicated by +, + +, or + + +. ob = spindle shaped cells (osteoblasts?).

It would appear that the calcium is removed from the bone into the extravascular soft tissues, where it is held, or that it is excreted by the intestines. Evidence of the former is lacking, but proof of colonic excretion is indicated. The calcium loosened from the skeletal storehouse does not accumulate in the blood. In other words, the usual criterion of parathyroid hormone action, namely the increase in blood calcium above normal levels, is lacking and the apparent inference made by Collip, Pugsley, Selye and Thomson [7] that bilateral nephrectomy does not influence hormone action is incorrect. Furthermore, the destructive lesions caused by overdosage of the normal animal with hormone were not observed by us in the ne-

phrectomized rat. Pugsley [13] has shown that the fecal excretion of calcium is increased following hormone administration, and we suspect that a greatly increased intestinal excretion, perhaps of the nature of a vicarious compensation, may rid the system of the calcium moved out of the bone. It has been established that the blood phosphorus rises rapidly after nephrectomy, and since the excretion of calcium may proceed more rapidly by the intestinal route than under normal conditions, these two conditions may affect the amount of calcium that accumulates in the blood.

Action of Calciferol on the Bilaterally Nephrectomized Rat

Calciferol * administered in single subcutaneous doses to rats that were not fed during the 48 hour experimental period gave either doubtful or indecisive increases in the serum calcium (average 12.69 mg. per cent in Rats 1–4, Table II), and the bone was devoid of any distinct osteoclastic bone resorption. Even though large quantities of calciferol were injected subcutaneously in divided doses into normal rats (Rats 5–6, Table II) no osteoclastic reaction was produced within 48 hours. The remaining rats in Table II were bilaterally nephrectomized. Of these, Rats 7 and 8 showed osteoclast reaction to 1 cc. of calciferol (920,000 I.U.), but Rat 9 revealed no excess of these cells. There was no rise in calcium values above normal. Triple subcutaneous doses of 0.5 cc. of calciferol in Rats 16–17 gave definite elevations of calcium and a slight to a considerable osteoclast reaction with bone resorption (Fig. 3). The average amount of resorption and osteoclastic reaction in the series of calciferol-injected nephrectomized rats was not so great as in the nephrectomized animals injected with hormone, but in individuals of the former group the resorption and associated osteoclastic reactions were pronounced (Figs. 2 and 3). The "spontaneous" fractures frequently seen in the rats injected with parathyroid extract were unusual in the animals injected with calciferol. The calciferol injections in Rats 18–20 were delayed 5 to 20 hours after nephrectomy. Phosphorus determinations of the blood serum of these three animals showed an average terminal value of 24.35 mg. per cent, and yet the calcium was maintained at the normal value or slightly above.

* The calciferol was generously supplied by Mead Johnson and Co.

Discussion

In a study of calcium and phosphorus metabolism an examination of both skeletal and soft tissues is useful for the reason that pathological quantities of calcium may become microscopically demonstrable in the form of calcification or resorption, but especially be-

Table II

Effect of Calciferol on the Serum Calcium and on the Femurs of Bilaterally Nephrectomized Rats *

Rat No.	Weight (individual or average)	Calciferol solution	Serum calcium (individual or average)	Femur
	gm.	*cc.*	*mg./%*	
1–4	144	0.5	12.69	Normal
5	178	0.5 x 3		Normal
6	114	0.5 x 3	12.82	Normal
7	231	0.5 x 2	9.67	oc + +
8	239	0.5 x 2	10.23	oc + +
9	283	0.5 x 2	9.75	Normal
10	159	0.5 x 3	15.04	oc + +
11	144	0.5 x 3	15.77	oc + +
12	142	0.5 x 3	17.30	oc +
13	143	0.5 x 3	15.68	oc ++
14	129	0.5 x 3	16.04	oc +
15	150	0.5 x 3	13.22	oc + +
16	154	0.5 x 3	17.06	oc + +
17	159	0.5 x 3	15.04	oc + +
18	118	0.5 x 3	13.18	oc +
19	289	0.5 x 3	12.55	oc + +
20	180	0.5 x 3	11.40	oc + +

* The calciferol was dissolved in corn oil and administered subcutaneously. Each 0.5 cc. contained 11.5 mg. calciferol and was equivalent to 460,000 international units of vitamin D. The multiple doses were spaced about 6–12 hours apart.

Rats 1–6 inclusive were not operated upon. In Rats 18, 19, and 20 the serum inorganic phosphorus was 23.86, 30, and 19.20 mg. per cent, respectively, at the end of 48 hours. All animals were sacrificed 48 hours after the operation.

cause cell reactions which are more or less characteristic may result from abnormal variations in the calcium and phosphorus content of the tissues. Of these cells the osteoclasts of the bone are often a conspicuous feature of resorptive processes. Not only is the origin of this cell in doubt but its function is uncertain. Many investigators regard the cell as the "bone" macrophage whose counterpart in the soft tissues is the common macrophage. Experimental evidence has shown that the latter produces at least one enzyme, a protease. We know of no proof that the osteoclast is the source of the calcium-

liberating property attributed to it by Collip and co-workers.[7] Neither do we see that Ham and Lewis [11] have shown that the osteoclast is not a potent factor in the type of resorption that is seen in the lesions that may be produced with parathyroid extract and calciferol. Regardless of what its function may be, the osteoclast furnishes important evidence of the presence or absence of the type of resorption that we are considering.

A comparison of parathyroid hormone and calciferol effects in the bilaterally nephrectomized rat reveals that although both the hormone and the calciferol loosen calcium and remove it from the bone, it is only calciferol that effects a hypercalcemia during our 48 hour experimental period. The explanation of this emphatic difference probably rests on the mechanisms involved. Brown and Shohl [14] and Watchorn [15] found that irradiated ergosterol decreased calcium excretion in the feces and increased urinary excretion. In a series of rats fed a calcium- and phosphorus-free diet Shelling [16] found that of the total calcium excreted 5 per cent was excreted in the urine and 95 per cent in the feces; while in another series of rats fed the same diet, except that large amounts of viosterol were added, he found that only 3 per cent of the calcium was excreted by the intestines and 97 per cent in the urine. Presumably in our bilaterally nephrectomized rats that were injected with calciferol the shutting off of all urinary excretion by removal of the kidneys, together with the calciferol action tending to lessen fecal excretion, accounts for the hypercalcemia observed. That both calcium and phosphorus may rise simultaneously to high levels in the nephrectomized animal is shown by our observations of nephrectomized rats,[4] and dogs [5, 6] that had been injected with calcium gluconate. In those experiments it was found that the rise in serum calcium was dependent on the introduction of a sufficient quantity of the calcium salt. In our series of calciferol-injected rats the calcium salts were drawn from the bone and the available evidence indicates that it accumulated in the blood because of decreased fecal excretion.

In the nephrectomized hormone-injected rat the situation is different. We think that it is quite probable that the amount of extraskeletal calcium liberated by the action of parathyroid hormone does not accumulate in the blood because much is eliminated by the intestinal route which is generally accepted to be the normal route. Pugsley,[13] using small doses of parathyroid extract, found an accen-

tuation of calcium excretion in the urine and feces. In the normal rat large subcutaneous doses of parathyroid extract are followed by hypercalcemia, although there is normal or excessive excretion of calcium by the intestines, but in the nephrectomized rat, in which there is a definite tendency for the phosphorus level to rise, and for the calcium to fall,[4] a characteristic hypercalcemia does not follow the administration of large doses of parathyroid extract.

Summary and Conclusions

1. In the bilaterally nephrectomized rat the administration of large doses of parathyroid extract does not produce a characteristic rise in serum calcium but does cause a pronounced osteoclastic resorption of bone.

2. In the bilaterally nephrectomized rat subcutaneous injection of calciferol causes a hypercalcemia and also osteoclastic bone resorption within 48 hours.

3. The above observations are made the basis for a discussion of the probable mechanisms of action of parathyroid hormone and calciferol under the experimental conditions described.

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DESCRIPTION OF PLATE

PLATE 56

FIG. 1. Photomicrograph from a section of metaphyseal end of femur showing resorption that followed in 26 hours after the injection of 750 (Hanson) units of parathyroid extract (Lilly) into nephrectomized Rat 16 (Table I). Numerous spindle shaped cells are apparent. Osteoclasts are present but not especially numerous. × 141.4.

FIG. 2. Photomicrograph from a section of metaphyseal end of femur showing the increased resorption of bone that followed in 48 hours after the administration of 1.5 cc. of calciferol (3 × 460,000 I.U.) to Rat 11 (Table II). Spindle shaped cells are less conspicuous than in the hormone-injected animal but osteoclasts are numerous. × 141.4.

FIG. 3. Photomicrograph from section of metaphyseal end of femur showing the increased resorption of bone that followed in 48 hours after the administration of 1.5 cc. of calciferol to nephrectomized Rat 17 (Table II). Here again the osteoclasts are active in the resorption. × 258.

1

2

3

McJunkin, Tweedy and McNamara Effect of Parathyroid Extract and Calciferol

PHAGOCYTIC ACTIVITY OF CIRCULATING CELLS IN THE VARIOUS TYPES OF LEUKEMIA *

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The purpose of this study was to determine the variations, if any, in the phagocytic activity of cells found circulating in the various forms of leukemia, as well as in normal blood, and thus to establish a physiological criterion for differentiation in addition to those well known morphologically. The work was carried on by the two workers on different cases at different institutions and employing differing methods.

STRUMIA'S METHOD

The cells are obtained by taking 5 to 20 cc. of blood from the vein and mixing it immediately with 50 to 200 cc. of the following solution: sodium chloride 8.5 gm., sodium citrate 10 gm., and distilled water 1000 cc. By careful centrifugation the buffy layer or leukocytic cream is separated and washed three times with salt solution, the final suspension containing from 40,000 to 50,000 leukocytes per cmm.

In all the experiments pooled, fresh, unheated serum from adults was used. The bacteria were from a strain of *Staphylococcus aureus haemolyticus* that has been used extensively in the laboratory because it is readily phagocytozed. The cocci were obtained from an 18 hour broth culture and were washed three times with salt solution. The final suspension was made to contain two billion bacteria per cc.

One-tenth cc. of serum diluted with salt solution, 0.2 cc. of white cells in suspension, and 0.1 cc. of bacterial suspension are mixed in small glass tubes and shaken once a minute for ½ hour at room temperature. Immediately before smears are made 1 drop of fresh serum is added to each tube and the contents mixed. Smears are then prepared on clean slides, air dried and stained by a modification of the May-Grünwald-Giemsa method.[1] At least 100 cells of each type are counted, noting both the number of cells showing phagocytosis and the total number of bacteria contained.

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All the experiments on the phagocytic activity of circulating leukocytes were done over a period of 3 years, using exactly the same technic and thus obtaining comparable results.

Table I summarizes the results obtained in five experiments in which normal leukocytes from adults were employed. This table shows that maximum phagocytosis was obtained with neutrophilic polymorphonuclears, that the monocytes were a close second, although constantly less active than the neutrophilic polymorphonuclears, both in percentage of cells showing phagocytosis and in the total number of phagocytozed bacteria. The eosinophiles show distinctly less phagocytic activity than either neutrophiles or monocytes, especially in the total number of ingested bacteria, and the lymphocytes show no phagocytosis at all.

Experimental

Acute Lymphatic Leukemia: The cells were obtained from Margaret B., a white female, aged 14 years. At the time the cells were obtained the white count was 46,700 with 91.5 per cent lymphocytic cells, of which nearly one-half were lymphoblasts. The results in Table II show clearly that neither the immature lymphocytes (lymphoblasts, prolymphocytes) nor the mature lymphocytes showed any phagocytosis, and that the young and mature neutrophiles showed phagocytic activity that may be considered close to the normal limits.

Chronic Lymphatic Leukemia: Mrs. Celia F., a white female, aged 60 years, presented a classical picture of the disease and at the time of the experiment showed 54,500 white cells per cmm. of blood, 96 per cent of which were lymphocytic, including 80.5 per cent prolymphocytes. None of the lymphocytic cells showed any phagocytic activity whatever, whereas the neutrophilic cells showed active phagocytosis (Table III). It may be stated here that in over 8 years of work on the problem of phagocytosis in which thousands of slides have been examined, phagocytosis by lymphocytic cells has never been observed, which seems remarkable if one considers that even accidentally bacteria might be expected to overlap occasionally on lymphocytes.

Acute Myelogenous Leukemia: Catherine R., a white female, aged 9 years. The white cell count at the time of the experiment was

TABLE I

Normal Blood — Average of Five Adults

	Neutrophilic polymorphonuclears				Eosinophiles				Monocytes				Lymphocytes			
Concentration of serum	1:16	1:64	1:256	C*	1:16	1:64	1:256	C	1:16	1:64	1:256	C	1:16	1:64	1:256	C
Percentage of cells containing bacteria	100	98	36	12	83	52	7	4	91	90	41	14	0	0	0	0
Total number of bacteria ingested by 100 cells	1105	457	48	17	257	86	8	4	835	783	115	21	0	0	0	0

* C = control (salt solution).

TABLE II

Acute Lymphatic Leukemia

	Oxidase-negative hemocytoblasts, lymphoblasts and prolymphocytes				Lymphocytes				Neutrophiles			
Concentration of serum	1:16	1:64	1:256	C	1:16	1:64	1:256	C	1:16	1:64	1:256	C
Percentage of cells containing bacteria	0	0	0	0	0	0	0	0	100	96	13	5
Total number of bacteria ingested by 100 cells	0	0	0	0	0	0	0	0	735	581	17	7

27,800, with myeloid hemocytoblasts or undifferentiated oxidase-positive cells 42 per cent, immature granulocytic cells (myeloblasts and promyelocytes) 16 per cent, and young granulocytes 18 per cent. The experiment showed that the hemocytoblasts and myeloblasts possessed but slight phagocytic activity; the metamyelocytes, rod nuclears and neutrophilic polymorphonuclears, or young granulocytic cells, showed a strong phagocytic activity, and that the lym-

TABLE III

Chronic Lymphatic Leukemia

	Lymphocytic cells				Neutrophiles			
Concentration of serum	1:16	1:64	1:256	C	1:16	1:64	1:256	C
Percentage of cells containing bacteria	0	0	0	0	100	100	16	6
Total number of ingested bacteria	0	0	0	0	1223	983	19	8

phocytic cells showed no phagocytic activity whatsoever (Table IV). This experiment was repeated with similar results. However, in the second experiment the phagocytic power of the leukemic cells was tested, using both the patient's serum and pooled serum from normal adults; also leukocytes from healthy individuals were tested for phagocytic activity with both the patient's serum and the same mixed adult normal serum. The conclusion on this second group of experiments was as follows: first that the predominating leukemic cells (undifferentiated and immature oxidase-positive cells) showed slight or doubtful phagocytosis, either with the patient's serum or adult mixed serum; second, that the patient's young and mature granulocytes (over 5000 per cmm.) were actively phagocytic with both serums; and third, that the patient's serum had a high opsonic index. Nevertheless, the patient had severe mouth ulcerations. Türck cells showed no phagocytic activity.

Chronic Myelogenous Leukemia: Agnes M., a white female, aged 59 years, with a white cell count of 309,000, offered a classical picture of chronic myelogenous leukemia, both clinically and hematologically. The granulocytic cells were divided into six groups and the phagocytic activity appeared to be distributed as follows: myelo-

TABLE IV

Acute Myelogenous Leukemia

	Oxidase-positive hemocytoblasts and myeloblasts				Neutrophiles				Lymphocytes			
Concentration of serum	1:16	1:64	1:256	C	1:16	1:64	1:256	C	1:16	1:64	1:256	C
Percentage of cells containing bacteria	6	2	0	0	100	99	41	16	0	0	0	0
Total number of ingested bacteria	11	2	0	0	1500	1210	391	60	0	0	0	0

TABLE V

Chronic Myelogenous Leukemia

Neutrophilic Cells

	Myeloblasts				Promyelocytes				Myelocytes				Metamyelocytes				Rod nuclears				Polymorphonuclears			
Concentration of serum	1:16	1:64	1:256	C	1:16	1:64	1:256	C	1:16	1:64	1:256	C	1:16	1:64	1:256	C	1:16	1:64	1:256	C	1:16	1:64	1:256	C
Percentage of cells containing bacteria	3	1	0	0	7	5	0	0	43	39	8	5	86	81	26	7	98	100	35	9	100	99	39	8
Total number of ingested bacteria	5	2	0	0	13	6	0	0	201	86	13	5	510	405	38	9	956	723	61	13	1015	810	70	14

blasts and hemocytoblasts, slight to doubtful; promyelocytes, slight; myelocytes, distinct phagocytosis; metamyelocytes, active phagocytosis; rod nuclears, active phagocytosis (more than the preceding group); polymorphonuclears, active phagocytosis (slightly more than preceding group). It appears, therefore, that all of the granulocytic cells show some degree of phagocytosis but that the appearance of maximal phagocytic activity is coincidental with the maturation of the cytoplasm and the appearance of the mature neutrophilic granulations (Table V).

Owing to the importance of these findings the experiment was repeated on another case of chronic myelogenous leukemia, Roy P., a white male, aged 29 years. The results fully confirmed the findings already stated. Basophilic cells show some phagocytic activity, but it appears to be much less than that of the neutrophiles. Note that it is difficult to see bacteria in the basophilic cells.

Acute Hemohistioblastic Leukemia: Robert C., a negro, aged 35 years, presented an unusual case of extremely rapid hemohistioblastic or acute monoblastic leukemia. Diagnosis was made from the blood study and from biopsy of the sternum during life, confirmed at autopsy. The white cell count was 51,300 at the time of the experiment, with 83 per cent hemohistioblastic cells. Study was made on cells from the circulating blood as well as from an emulsion of cells from the sternum. The results showed that the hemohistioblastic cells have a strong phagocytic activity and that the monoblasts also have phagocytic activity but somewhat less than that of the hemohistioblastic cells (Table VI). The few polymorphonuclears present in this case also showed active phagocytosis. The finding that these highly undifferentiated cells behave in an entirely different fashion from the leukemic cells of other types is in full accord with the morphological implications. An emulsion of cells from the sternal marrow, obtained during life, showed that the hemohistioblastic cells predominated and that they were actively phagocytic toward a variety of particles, including bacteria, erythrocytes, carbon and collodion particles. This cellular emulsion was not washed. It contained an unknown quantity of the patient's serum and therefore results from this experiment are not strictly comparable. However, it appeared that collodion particles, large and small, and to a lesser degree carbon particles, were more actively phagocytozed by the hemohistioblastic cells than by neutrophilic polymorphonuclears.

TABLE VI

Acute Hemohistioblastic (Monoblastic) Leukemia

	Hemohistioblasts				Monoblasts				Neutrophiles			
Concentration of serum	1:16	1:64	1:256	C	1:16	1:64	1:256	C	1:16	1:64	1:256	C
Percentage of cells containing bacteria	87	49	12	15	63	25	7	9	100	73	9	11
Total number of ingested bacteria	515	408	13	15	290	128	7	10	810	343	10	11

TABLE VII

Acute Hemohistioblastic (Monoblastic) Leukemia

	Hemohistioblasts, monoblasts and monocytic cells				Immature granulocytic cells (myeloblasts and promyelocytes)				Mature granulocytic cells			
Concentration of serum	1:16	1:64	1:256	C	1:16	1:64	1:256	C	1:16	1:64	1:256	C
Percentage of cells containing bacteria	61	48	21	23	7	2	0	0	94	81	18	11
Total number of ingested bacteria	405	301	63	48	11	2	0	0	883	629	31	15

These findings are particularly significant as they parallel differences in behavior of rabbits' exudative macrophages (monocytic cells) and polymorphonuclears toward small collodion particles.[2] Rieder cells of this same case appear to possess phagocytic power, approximatively 30 per cent of them containing bacteria.

Acute Hemohistioblastic Leukemia: George H., a white male, aged 4½ years, showed a most unusual type of acute leukemia of the hemohistioblastic type (monoblastic) with a severe pneumonitis, probably terminal, and a large number of immature granulocytic cells. The white count at the time of the experiment was 131,000. Experiments again showed active phagocytosis by the hemohistioblastic, monoblastic and monocytic cells, slight phagocytosis by the immature granulocytic cells, including myeloblasts and promyelocytes, and the usual maximal phagocytic activity by the mature and young granulocytic cells including metamyelocytes, rod nuclears and polymorphonuclears (Table VII).

Glandular Fever: We also investigated the phagocytic activity of cells from a case of glandular fever (acute mononucleosis) which, although not connected with the leukemias, offers occasionally to the untrained some difficulty in the differential diagnosis. Joan G., a white female, aged 14 years, had at the peak of the attack a white cell count of 14,100, with the following differential count: rod nuclears 16, polymorphonuclears 41, eosinophiles 1, monocytes 3, prolymphocytes 1, lymphocytes 23, leukocytoid lymphocytes 3, and abnormal degenerated lymphocytic cells 12. It was particularly to test the phagocytic activity of this last group that the experiment on phagocytosis was performed. These cells are commonly classed as monocytes, although accurate morphological studies show them to be abnormal lymphocytes.[3] Strumia considers these cells degenerated forms of immature lymphocytic cells (lymphoblasts) but does not admit any relation to plasma cells. Phagocytosis experiments showed that these cells have no phagocytic activity whatever, giving further support to the view that these cells are lymphocytic.

Boerner's Method

This method is the same as that described by Boerner and Mudd,[4] and the results are summarized in Table VIII. One cc. of heparinized blood is placed in short test tubes (15 by 5 mm.) and placed in

TABLE VIII

TABLE VIII

Acute and Chronic Myelogenous Leukemia, Chronic Lymphatic Leukemia and Allergic Conditions

Case No.	Diagnosis	Organism	Serum	Hemocytoblasts	Myeloblasts	Promyelocytes	Myelocytes	Metamyelocytes and rod nuclears	Polymorphonuclear neutrophiles	Eosinophiles	Lymphocytes	Monocytes
1	Acute myelogenous leukemia	Pneumo. 1 R	0	0					92			
		" "	1:50	2					94			
		Staph. albus	0	0					86			
2	Acute myelogenous leukemia	Staph. albus	0		1.5				100			
		" "	1:10		1				100			
		Pneumo. 1 R	0		4				99			
		" "	1:50		2				99			
3	Chronic myelogenous leukemia	Pneumo. 1 S	1:50		0	0	40	50	74			
		" 1 R	0		0	0	43	58	71			
		" 1 R	1:50		0	0.16	85	90	98			
		Staph. aureus	0		0	0	45	70	72			
		" "	1:50		0	0	40	53	72			
4	Chronic myelogenous leukemia	Staph. aureus	0			0	50	50	65			
		" "	1:50			2	53	62	70			
		Pneumo. 1 R	0			6	64	90	88			
		" "	1:50			4	57	90	94			
5	Allergic condition	Strep. Hem. (mucoid)	0						72	40		
		Strep. Hem. (glossy)	0						98	87		
		Pneumo. 1 S	1:10						95	15		
		" "	0						13	8		
		Yeast	0						14	8		
		Erythrocytes	0						44	3		
6	Allergic condition	Pneumo. 1 S	0						26	4		
		" 1 S	1:75						100	64		
		Staph. aureus	0						100	100		
7	Chronic lymphatic leukemia	Staph. aureus	0						100		0	94
		" albus	0						100		0	86

Figures represent percentage of cells showing phagocytosis.

the agitator bath kept at a temperature of 37° C. In some instances 0.1 cc. homologous serum was added to the heparinized blood. To the blood or blood-serum mixture 0.1 cc. bacterial suspension in salt solution is added, containing 6 billion bacteria per cc., then after 15 minutes agitation smears are made and stained with Giemsa's solution. The antipneumococcus serum was obtained from the horse; the antistaphylococcic serum was of human origin. In counting, the percentage of cells showing ingested bacteria is noted.

CASE 1. Jeannette K., a white female, aged 17 years, suffering from acute myelogenous leukemia, showed at the time of experiment a leukocyte count of 12,000, with 75 per cent hemocytoblasts, the majority of which were oxidase-positive. The hemocytoblasts showed slight to doubtful phagocytic power; the mature polymorphonuclears active phagocytosis. In this experiment pneumococci and *Staph. albus* were employed.

CASE 2. Emma L., a white female, aged 60 years. Another case of acute myelogenous leukemia with a leukocyte count of 126,000 per cmm. at the time of experiment. The differential count showed a distinct prevalence of typical myeloblasts, forming with the more undifferentiated hemocytoblasts 82 per cent of the total circulating leukocytes. The myeloblasts showed a slight but definite phagocytic activity. The mature neutrophiles showed an unusual maximal phagocytic activity. In this experiment pneumococci and *Staph. albus* were employed.

CASE 3. Mrs. Ethel C., a white female, aged 40 years, with chronic myelogenous leukemia, previously treated with X-ray. In this experiment the myeloblasts showed no phagocytic activity, the promyelocytes slight phagocytosis, the myelocytes, metamyelocytes and rod nuclears and the mature polymorphonuclears showed active phagocytosis, increasing slightly but definitely in each successive group in the order mentioned. In this experiment pneumococci and *Staph. aureus* were employed.

CASE 4. Roy P., a white male, aged 29 years, a case of long standing (7 years) chronic myelogenous leukemia, repeatedly treated with X-ray. About the time of experiment blood examinations showed a leukocyte count of over 100,000 per cmm., with about 80 per cent promyelocytes and all forms of granulocytic cells represented. Experiments employing *Staph. aureus* and pneumococci showed slight but definite phagocytosis with promyelocytes, active phagocytosis

for myelocytes, metamyelocytes and rod nuclears, and polymorphonuclears, with slight increase in the order given. Cells from Cases 3 and 4 showed closely similar behavior.

CASE 5. Horace L., a white male, aged 25 years, convalescing from asthma and colitis of allergic nature. This patient at the time of experiment had a total leukocyte count of 12,200, of which 28 per cent were neutrophiles and 57 per cent eosinophiles. This afforded a good opportunity for a comparative study of the two types of cells, with a variety of particles (two strains of hemolytic streptococcus, two of pneumococcus, yeast cells and erythrocytes). The eosinophiles showed active phagocytosis, but always considerably below that of neutrophiles, especially for certain type of particles.

CASE 6. Mary W. H., a white female, aged 44 years, presenting chronic follicular conjunctivitis showing on the day of experiment a leukocyte count of 8200 with 55 per cent neutrophiles and 19 per cent eosinophiles. With pneumococci phagocytosis appeared definitely less with the eosinophiles than with the neutrophiles. With a strain of *Staph. aureus* the percentage of phagocytosis was maximal with both types of cells, but the total number of bacteria ingested by the eosinophiles was far below that ingested by the same number of neutrophiles (see Table IX). This is also apparent from Table I giving data from normal blood.

CASE 7. Harry D., an adult white male, suffering from chronic lymphatic leukemia treated with X-ray. Prior to experiment blood examinations had shown a leukocytic content of over 200,000 per cmm., with a predominance of young lymphocytic cells (prolymphocytes). Experiment showed a maximal phagocytosis with the neutrophiles, only slightly less active phagocytosis with monocytes, and no phagocytosis with any of the lymphocytic cells. *Staph. aureus* and *Staph. albus* were used in this experiment.

DISCUSSION

Making due allowance for the great variation in the state of preservation of leukemic cells, for the difference in the methods used in the two series, and for the difference in the particles employed, the results are remarkably uniform. In the first series, using Strumia's method, the washing of the cells has the great advantage of eliminating the variations of the opsonic value of the patient's serum,

TABLE IX

Case 6

Pneumococcus Type 1 S

	No. bacteria per cell	Time in minutes				
		3	6	9	12	15
Eosinophiles	over 10	0	0	0	0	0
	5 to 10	0	0	0	0	0
	1 to 5	1	6	3	8	4
Neutrophiles	over 10	1	1	0	1	0
	5 to 10	2	1	0	0	1
	1 to 5	18	13	8	12	26

Pneumococcus Type 1 S

Antipneumococcus serum (1:75)

Eosinophiles	over 10	6	8	11	17	10
	5 to 10	10	12	11	16	18
	1 to 5	28	35	29	31	36
Neutrophiles	over 10	30	65	90	93	88
	5 to 10	20	19	5	5	4
	1 to 5	20	15	3	2	8

Staphylococcus aureus

Eosinophiles	over 10	23	68	68	72	80
	5 to 10	36	16	15	13	10
	1 to 5	35	12	13	12	10
Neutrophiles	over 10	66	100	99	100	100
	5 to 10	28	0	0	0	0
	1 to 5	6	0	1	0	0

substituting progressive dilution of a serum pooled from ten or more patients, which has proved to be remarkably constant in its opsonic value, at least with the strain of staphylococcus used, thus affording a comparable series of cases. The counting of the actual number of bacteria phagocytozed in addition to the percentage of cells showing phagocytosis, affords a much better quantitative measure of the phenomenon. A disadvantage of the method is the greater time required and the greater chance of cell damage. Both of these factors can be reduced to a minimum by carefully following the technic.

The advantages of the method of Boerner and Mudd are its simplicity of technic, the small amount of blood required, and above all that the leukocytes have undergone only a minimum of manipulation and are functioning in their natural medium. In later work with this method the number of bacteria per cell, as well as the percentage of phagocyting leukocytes has been counted. The disadvantage of the method is the undetermined variation of the opsonic index of the patient's serum, which renders the data less reliable for comparative study.

Although leukemic cells are probably not identical in function with normal cells, they are similar enough so that conclusions concerning their phagocytic activity may be expected roughly to apply to normal cells of corresponding development. This statement is based on comparative studies made by one of us (Strumia) although not enough experimental data are on hand to justify final conclusions. As the work stands, the phagocytic activity of mature polymorphonuclears and eosinophiles from fresh human rib marrow emulsion is comparable to that of the same cells from circulating blood, although usually less. Lymphocytic cells from lymph nodes of rabbits, and lymph nodes removed at autopsy from children within 6 hours of death, show no phagocytosis. Monocytic cells from a splenic emulsion (from an accident case within 3 hours of death) freed as much as possible of blood, show active phagocytosis. Many of these cells, however, appear to be younger than the circulating monocytes (monoblasts and hemohistioblasts).

However, when we wish to study young and immature cells from a non-leukemic subject the variations are greater. Thus, immature and young granulocytic cells from fresh human rib marrow have usually less phagocytic activity than the corresponding leukemic circulating cells; whereas circulating young granulocytic cells,

metamyelocytes and rod nuclears, from cases of infections with mild toxemia, show generally more phagocytosis than the corresponding leukemic circulating cells.

It is essential to avoid cell injury as much as possible and, in studying circulating leukocytes in cases of infections, to use only patients whose blood shows on ordinary stained smears no signs of severe degenerative changes of the leukocytes.

Summary and Conclusions

Lymphocytic cells never show phagocytosis. Undifferentiated oxidase-negative cells (lymphoid hemocytoblasts) likewise show no phagocytic activity. Granulocytic cells and their precursors constantly show active phagocytosis, as follows: undifferentiated oxidase-positive cells (myeloid hemocytoblasts) show slight phagocytic activity in some experiments, doubtful in others. The immature cells (myeloblasts and promyelocytes) show a slight but definite and progressive increase in the phagocytic activity. It is to be noted that the promyelocyte, which is the first cell of the granulocytic series to show the appearance of the specific neutrophilic (or eosinophilic) granulation, is also the first cell showing a definite phagocytic activity in all experiments conducted.

The myelocyte, the first cell to possess an entirely mature cytoplasm, namely an acidophilic cytoplasm, with mature (neutrophilic) granulations, shows phagocytic activity greatly increased over the preceding cells. There is further increase in the phagocytic activity with the other granulocytic type of cells, namely metamyelocytes, rod nuclears and polymorphonuclears. The possible relation of phagocytosis to the area of cytoplasm was considered and investigated. The phagocytic activity of the myelocyte, metamyelocyte, rod nuclear and mature polymorphonuclear obtained from a number of experiments on leukemic and non-leukemic blood, compared with the relative area of the cytoplasm in smears of these cells, can be summarized as follows:

Neutrophilic cells	Phagocytic activity*	Area of cytoplasm exclusive of nucleus†
Myelocytes	30	86
Metamyelocytes	67	90
Rod nuclears	96	97
Polymorphonuclears	100	100

* Per cent of cells showing phagocytosis.
† Relative surface of cytoplasm on stained preparations, taking that of the neutrophilic polymorphonuclears as equaling 100.

In the above table, while for the myelocyte and the metamyelocyte there is no parallelism between the surface of the cell cytoplasm and its phagocytic activity, a parallelism between the two figures exists in the rod nuclear and the polymorphonuclear. It is interesting to note that the rod nuclear only of the young cells is normally present in circulation. One may conclude that in so far as the phagocytic activity is concerned there is no difference between the mature polymorphonuclear and the rod nuclear.

Eosinophiles show phagocytic activity but far less than either the neutrophiles or the monocytes. It is to be noted that while a fair percentage of eosinophiles shows phagocytosis, each cell usually contains fewer bacteria. This can readily be seen from Table I (normal blood), and from Table VIII. The basophiles show even less phagocytic activity, but allowance must be made for the fact that it is difficult to differentiate between granules and bacteria.

Monocytic cells (hemohistioblasts, monoblasts, monocytes) constantly show active phagocytosis. In so far as the bacteria employed are concerned monocytes appear somewhat less active than neutrophilic polymorphonuclears, but collodion granules and carbon particles appear to be picked up more readily by monocytic cells. Rieder's cells show slight but definite phagocytic activity; Türck's cells show none.

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DESCRIPTION OF PLATE

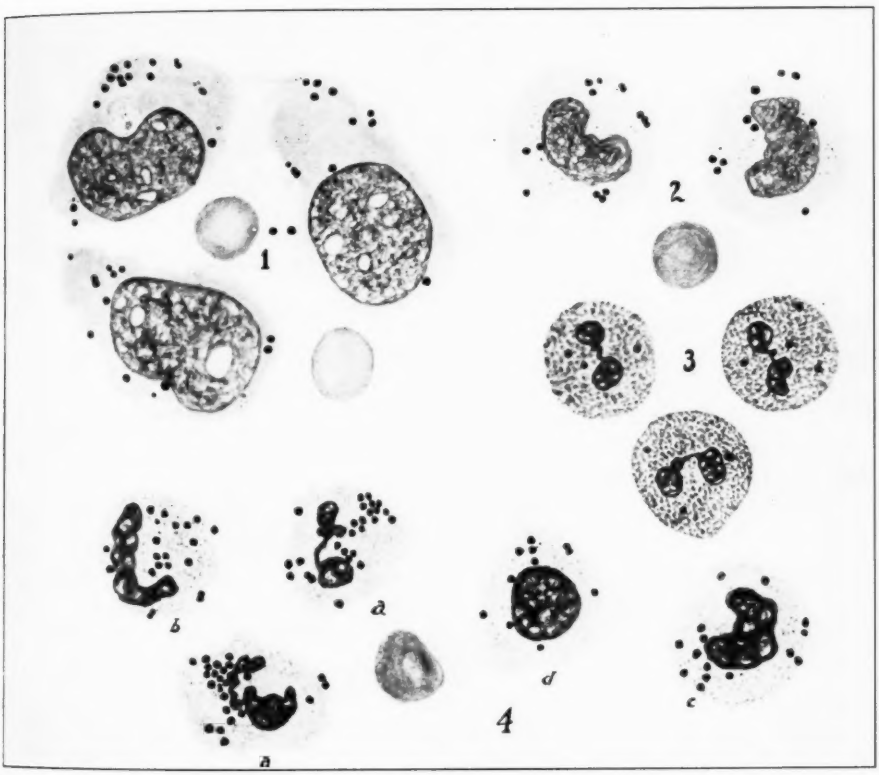
PLATE 57

FIG. 1. Hemohistioblasts from a case of acute hemohistioblastic (monoblastic) leukemia showing phagocytosis of staphylococci (Strumia's method).

FIG. 2. Monocytes from normal circulating blood with phagocytozed staphylococci.

FIG. 3. Eosinophiles from normal circulating blood with phagocytozed staphylococci.

FIG. 4. Neutrophilic cells from a case of chronic myelogenous leukemia showing phagocytozed staphylococci. a = mature polymorphonuclears; b = rod nuclear; c = metamyelocyte; and d = myelocyte.



Strumia and Boerner Phagocytic Activity of Circulating Cells

THE PANETH CELL *

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In the small intestine of man and many animals there exists at the base of the crypts of Lieberkühn a striking eosinophilic cell. Schwalbe,[1] in 1872, was the first to describe these cells. It remained for Paneth,[2] in 1888, to bring them to the attention of the investigators and for this reason they are known as Paneth cells. Paneth described them at some length and as a result of his efforts they became recognized as constant constituents of the intestinal glands of certain animals. Paneth regarded these granular cells as a specific kind of gland cell wholly different from the goblet cell. In 1892 Bizzozero [3] claimed to have found in material from the intestine of the mouse transitional forms between the Paneth cell and the goblet cell. He assumed, therefore, that the Paneth cell was a young goblet cell. Möller,[4] in 1899, found Paneth cells in the intestinal glands of the guinea pig, rabbit, ox, sheep and horse. He was unable to demonstrate them in the pig, cat and dog. His observations led him to conclude that the Paneth cell possessed a true secretory function. In 1906 Klein [5] found basal filaments in the cytoplasm of Paneth cells of the guinea pig. He found that the cells responded to physiological stimulation, which led him to consider that they represent a zymogenic cell involved in digestion. In 1905 Schmidt [6] studied the distribution of the cells of Paneth in man and confirmed Bloch's [7] observation (1903) that they occurred in practically every gland of the ileum and jejunum as well as the duodenum. In addition, Schmidt found them present frequently in the glands of the appendix, although he was not able to find them in other portions of the large intestine, except in 3 cases in which pathological conditions were present. Concerning their occurrence in the large intestine of infants, where Bloch claims to have observed them, Schmidt recorded a negative result in five newborn infants. For the differentiation between goblet cells and Paneth cells he used mucicarmine. Prenant,[8] in 1907, because of a similarity in staining of the goblet cell and the

* This work was started while the author was a Fellow in Pathology, The Mayo Foundation, Rochester, Minnesota.

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Paneth cell with a "vert-lumière" stain, concluded that the Paneth cells were mucus cells but that they represented specific types that were independent of the goblet cells. Bensley,[9] in 1908, held that Prenant's "vert-lumière" stain was not a specific stain for mucus, and in view of his observations concluded that the Paneth cell resembles a zymogenic rather than a mucus cell. Stöhr and Lewis,[10] in 1913, reported Paneth cells as occurring in the duodenum, jejunum and ileum of man but not in the large intestine. They said that the Paneth cell probably produces a special secretion which enters the lumen of the gland in the form of fine granules when the digestion of fat is taking place and that it may perhaps also be concerned with protein, but not with carbohydrate digestion. Chuma,[11] in 1923, in a study of the gastric mucosa in both normal and pathological conditions, mentioned the occasional occurrence of Paneth cells in pathological gastric conditions. Clara,[12] in 1926, concluded that the Paneth cell, although it did not normally arise from the principal epithelial cells of the mucosa in man, might originate from these cells under certain conditions, since it is known that in cases of chronic intestinal irritation, such as produced by carcinoma, the number of Paneth cells might be increased. He also reported their occurrence in the large intestine of domestic animals. Mols,[13] in 1930, studied the effects of different diets on the Paneth cells of mice. He concluded that the Paneth cells are a type of serous cell. Baecker,[14] in 1934, wrote an extensive article on Paneth cells and summed up their occurrence in various forms of animal life. He expressed the opinion that these cells were widely distributed in ancestral forms of life and gradually became localized in the crypts. He also said that the Paneth cell and goblet cell are closely related but that the Paneth cell, because of its wide distribution in nature, constitutes a definite cell type. He was unable to find in lower animal life a predecessor for both the Paneth cell and the goblet cell. Cowdry [15] considered the possibility that the Paneth cells and the enterochromaffin cells might be the source of the duodenal hormones, secretin and cholecystokinin. However, he concluded that this is highly improbable as they are found in regions of the intestine that do not manufacture hormones; also, the Paneth cell looks like an exocrin cell discharging its granules directly into the lumen of the crypts. He concluded by saying that what they produce is a mystery as the reports following the use of different diets are conflicting.

Boerner-Patzelt,[16] in 1935, studied the influence of changing the pH of the fixative on the staining characteristics of the granules of the Paneth cells. By regulating the pH of a concentrated aqueous solution of picric acid and using a stain composed of methylene blue and kreseponceau, some of the granules in the same cell took the blue stain while others took the red stain. She thought this variation in staining depended on the age of the secretion granules. She concluded that since the isoelectric points of the Paneth cell and goblet cell do have the same pH, a close relation between the two cells does not seem improbable.

In view of the many conflicting reports concerning these cells and the fact that the majority of the work done has concerned their occurrence in lower animal life, an investigation under normal and pathological conditions in human beings seemed in order.

Material

The greater part of the material used for this study was obtained at fifty routine autopsies. The sections from the intestine were secured as soon as possible at the autopsy table and placed in Orth's fixing fluid. The routine stain used was hematoxylin and eosin. In addition, special fixatives and stains were used, which will be described later. In order to study fresh material several sections of small intestine were secured from the operating room. Because of the difficulty in securing fresh human material, additional material was secured from guinea pigs that had been fasting for 12 hours. The time that elapsed between death and autopsy varied from 1 to 7 hours. In cases in which more than 7 hours had elapsed between death and autopsy the material usually was not suitable for routine study, and in many cases in which less than 7 hours had elapsed following death the material had to be excluded because postmortem changes made cytological studies impossible.

Morphology

The Paneth cell is a flask shaped cell with a broad base in contact with the basement membrane of the crypt of Lieberkühn and a narrow apex that reaches the lumen of the crypt. The cell possesses a round spheroidal nucleus, which is poor in chromatin and is situated near the base of the cell. Usually a distinct nucleolus is seen. The cytoplasm is light staining and the distal portion above

the nucleus is filled with large, round, brilliant eosinophilic granules which are all approximately the same size (Fig. 1). It is interesting to note that no granules were ever seen outside of the cell within the lumen of the crypts.

Situation and Distribution Under Normal and Pathological Conditions

In the small intestine of man the Paneth cells are seen at the very base of the crypts of Lieberkühn. They fill the distal ends of the crypts. When the lateral walls of the base of the crypts are examined goblet cells are found lying in apposition to the Paneth cells. The incidence of goblet cells then increases as the villi are approached. Kaufmann-Wolf,[17] in 1911, reported the occasional occurrence of Paneth cells in the villi of the small intestine, but I was not able to confirm this finding. In this series of 50 cases Paneth cells were found within the crypts of every section of the small intestine examined, except that of a $4\frac{1}{2}$ months intrauterine fetus, which contained goblet cells but no Paneth cells.

In order to study the normal incidence of Paneth cells among the crypts of the different portions of the small intestine of man a count was made with the material obtained from these 50 cases. This was done by counting twenty crypts in sections obtained from the duodenum, jejunum and ileum of each of the 50 cases. The count was made in the field that represented the most typical structure of the section. Out of the twenty crypts counted the number containing Paneth cells was noted. Hence, if ten of the twenty crypts contained Paneth cells the incidence in that section would be 50 per cent. The incidence in the different portions of the small intestine was as follows: in the duodenum, 48.5 per cent; in the jejunum, 74 per cent; and in the ileum, 73.5 per cent. The Paneth cells, therefore, seem to be most numerous in the jejunum and ileum, where their number is practically equal. They are found here in approximately three of every four crypts. In the duodenum their number is definitely less and they are found in approximately two of every four crypts.

Paneth cells do not normally occur in the stomach. I am indebted to Dr. Charles Baker,[18] who recently made an interesting morphological study of the gastric mucosa and whose material I had an opportunity to examine. Paneth cells were not found in the normal stomach. In 50 per cent of a series of 100 cases of ulcer and

carcinoma of the stomach, abnormal intestinal-like mucosa was found in some regions. When this type of mucosa existed Paneth cells were found at the bottom of the crypts in 98 per cent of the cases (Fig. 2).

Paneth cells occasionally occur in the large intestine, including the appendix. The appendix and colon offered an opportunity to study further the effect of abnormalities of the mucosa, as found in inflammation, malignant conditions and granulomatous lesions, on the occurrence of Paneth cells.

In a study of the appendix Paneth cells were present in two of twenty-five apparently normal appendixes. Paneth cells were present in three of twenty-five diseased appendixes. Hence, inflammation seems to have little effect on the presence of Paneth cells in the appendix. Moreover, when they were present in the appendix their incidence was much less than it was in the small intestine.

The 50 cases in which material was obtained from the cecum, colon and rectum were divided into two groups. The first group includes 25 cases in which the large intestine appeared to be normal. Paneth cells were not found in the large intestine in this group of cases. The second group consists of 25 cases in which the large intestine was the site of a malignant growth, tuberculosis or chronic ulcerative colitis. Paneth cells were found in 3 cases in the latter group; 2 of these cases were tuberculosis of the cecum, and the 3rd was a case of chronic ulcerative colitis. In the cases in which Paneth cells were present their incidence was similar to that in the appendix, that is, it was less than the normal occurrence as established for the normal small intestine.

It is interesting to note that when Paneth cells exist in the stomach or colon in association with any malignant condition, usually the growth is strongly eosinophilic, staining intensely with eosin at the expense of the basophilic structures. There is also much condensed mucus present.

The subjects of this study ranged from a $4\frac{1}{2}$ months intrauterine fetus to an adult of 80 years. Age did not seem to have any influence on the occurrence of Paneth cells except in the $4\frac{1}{2}$ months fetus, in which case goblet cells but no Paneth cells could be demonstrated in the small intestine. Specimens were obtained from two infants who were born prematurely at the 7th and 8th months of pregnancy respectively. In both of these cases autopsy, which was performed

soon after death, revealed that Paneth cells and goblet cells were present in normal quantities. Sex did not seem to have any influence on the occurrence of these cells.

Paneth cells do not occur normally outside of the gastro-intestinal tract, but a Paneth cell was found in the pancreatic duct near its distal end. In the guinea pigs studied the Paneth cells showed little difference, other than in size, from those that occur in human beings.

Fixatives

In order to determine the effect of different fixatives on the Paneth granules separate sections of the small intestine of a guinea pig were placed in Bouin's, Orth's, Zenker's, Helly's and Bensley's fixatives, in absolute alcohol, and in a 10 per cent solution of formalin. They were then stained with hematoxylin and eosin. The effects of these fixatives on the Paneth granules varied only slightly. One, therefore, can conclude that the ordinary fixatives preserve the granules and that the choice of a fixative is determined largely by the staining method to be used. However, the best results as far as the distinctiveness and brilliance of the granules were concerned were obtained with Bouin's fixative.

Staining

The Paneth granules stain well with acid dyes such as eosin and acid rubin. Various differential stains have been used to study these cells. Mucicarmine fails to stain the Paneth granules but, as would be expected, it stains the mucus within the goblet cells a brilliant red color. The Paneth granules are not stained by silver salts. An attempt was made to demonstrate a staining similarity between these cells and such substances as corpora amylacea of the prostate gland, lungs and central nervous system, and chondroproteins such as amyloid and similar substances. The results were pleasing. Congo red, a stain commonly employed in the study of amyloid material, brings out the Paneth granules distinctly, much more so than does eosin (Fig. 1). At times it is extremely difficult to say whether a section contains Paneth granules or not, with the use of hematoxylin and eosin, especially when the section is heavily stained with eosin. Congo red is particularly useful in these cases.

Paneth granules are stained a brilliant green color by de Galan-

tha's [19] amyloid stain, which contains carbol fuchsin, orange S, indigo carmine and picric acid. In an attempt to find Bizzozero's [20] transitional forms of Paneth cells, which contain both Paneth granules and mucus, it was noticed that the sections stained with ordinary hematoxylin and eosin did not have sufficient color contrast. Hence, a method was sought that would stain the Paneth granules one color, and at the same time stain the mucus within the goblet cells another. De Galantha's amyloid stain, when combined with mucicarmine, was found very satisfactory as the Paneth granules were stained a bright green and the mucus within the goblet cells a brilliant red. On careful examination of the crypts of Lieberkühn stained by this method it was found that one or two cells of a section situated at the base of the crypts contained numerous Paneth granules stained green and, in addition, red-staining droplets of mucus were present near the distal end of the cell. This finding was so striking that twenty sections of human small intestine stained with this technic were studied. The Paneth cells and goblet cells seemed to be independent of each other, but diligent search would show that from one to three cells of the section, which were situated at the base of the crypts, contained both the green granules and the mucus droplets. However, this was not found in every case studied as 7 of the 20 cases examined failed to reveal this phenomenon.

Best's carmine stain was first tried on human material that had been secured at autopsy and fixed in absolute alcohol. The results were negative. However, when the procedure was repeated on fresh specimens obtained from guinea pigs the Paneth granules gave a positive reaction for the presence of glycogen. Meriwether [21] has shown that corpora amylacea, both in the prostate gland and in the central nervous system, when stained with this stain, are found to contain glycogen.

Comment

The question is, what is the nature of the Paneth cells? The literature is divided into two schools: one considers the Paneth cell an independent zymogenic cell that possesses some unknown function; the other thinks it is closely related to the mucus cells of the intestine. Everything else being equal, it is easier to think of the Paneth cell as probably representing a cell closely related to the mucoid goblet cell rather than as an independent zymogenic cell.

Definite staining similarities exist between the Paneth granules and substances such as corpora amylacea of the prostate gland and amyloid material. The presence of Paneth cells out of their normal habitat, associated with pathological conditions as in carcinoma of the stomach, is extremely significant. When these cells are found associated with a carcinoma of the stomach there is usually a large quantity of coagulated mucus present. This would indicate to me that the Paneth cells are closely related to the production of mucus. Finally, de Galantha's stain demonstrates an occasional Paneth cell that seems to contain droplets of mucus as well as Paneth granules. However, this is not constant but could be demonstrated in a few crypts in 13 out of 20 cases observed. Bizzozero claimed to have found the same phenomenon and advocated the theory that the crypts of Lieberkühn acted as regenerative foci and that the Paneth cell is an early form of mucus cell. Prenant regarded these cells as mucus cells but of a different type from the goblet cell, as the Paneth cells are not stained by mucicarmine. If the Paneth cells are mucoid in nature their exact relation to the goblet cell is difficult to state, as today investigators are still somewhat uncertain as to the origin of the mucoid goblet cell. The Paneth cell could be regarded as a mucoid cell independent of the goblet cell, or as a primitive type of goblet cell. However, the exact nature of the Paneth cell will probably remain unknown until more definite microchemical reactions are utilized.

Conclusions

1. Paneth cells are coarsely granular cells found constantly in the small intestine of man.

2. They are most numerous in the jejunum and ileum, where their number is practically equal. They occur less frequently in the duodenum.

3. Paneth cells may occur occasionally in the colon and appendix, and also in the stomach, under pathological conditions.

4. Age and sex do not influence their occurrence.

5. The granules are easily preserved by the ordinary fixatives.

6. The Paneth cell gives evidence of being mucoid in character rather than an independent zymogenic cell.

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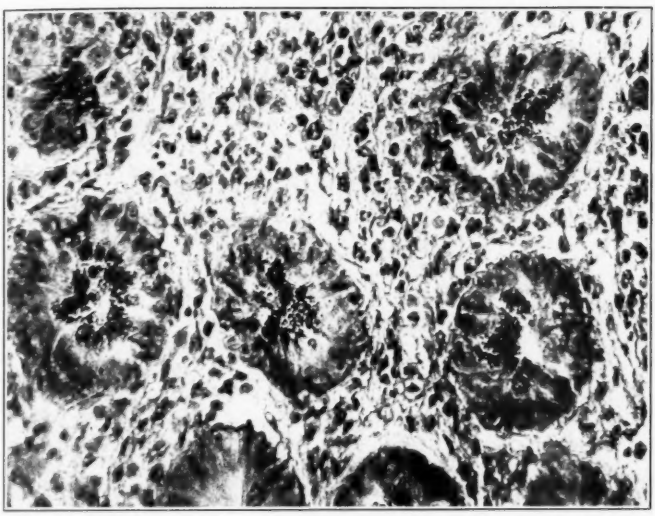
DESCRIPTION OF PLATE

PLATE 58

FIG. 1. Human ileum. Bouin's fixation, Congo red stain. × 415.

FIG. 2. Carcinoma of human stomach. The mucosa adjacent to the malignant cells is of the intestinal type; green-staining Paneth granules are present in these crypts. A large amount of mucus is associated. Orth's fixation, de Galantha's orange S plus Mayer's mucicarmine stain. × 70.



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Hertzog The Paneth Cell

UNIV. OF MICH.

CULTIVATION OF THE VIRUS OF ST. LOUIS ENCEPHALITIS *

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Among the various methods of tissue culture that have been adapted to the cultivation of viruses *in vitro* the techniques devised by Maitland and Maitland [1] and by Li and Rivers [2] have been widely employed for this purpose. Using similar conditions of cultivation Syverton and Berry [3] have recently demonstrated growth of the virus of St. Louis encephalitis [4, 5] in a medium composed of minced mouse embryo tissue, rabbit serum and Tyrode's solution.

The experiments to be reported in the present paper confirm the observations of Syverton and Berry and, in addition, reveal that the virus may be propagated in media containing chick embryo tissue, as well as in developing eggs, by the method of Woodruff and Goodpasture.[6]

MATERIALS AND METHODS

Strains of Virus

Three strains of the virus employed by one of us (E. M.) in previous experiments [7] were used in the present study; two (Daily and Barnes) were originally isolated by Muckenfuss, Armstrong and McCordock,[4] and the other (No. 3) by Webster and Fite.[5] Infected mouse brain, frozen and dried 7 months previously, was inoculated intracerebrally into mice, and the supernatant fluid of a 10 per cent brain emulsion from the 3rd mouse passage was used to initiate the cultures.

Methods of Cultivation

In preliminary experiments on cultivation of the virus a study was made of *in vitro* and *in vivo* methods. The media chosen for use were: A, that described by Syverton and Berry,[3] composed of finely minced living mouse embryo tissue suspended in fluid consisting of 1.7 cc. of Tyrode's solution and 1 cc. of fresh normal rabbit serum, and distributed in 50 cc. Erlenmeyer flasks; B, similar medium con-

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taining 2.7 cc. of Tyrode's solution without serum; C, minced chick embryo tissue, Tyrode's solution and rabbit serum in the same proportions as in medium A; and D, chick embryo tissue in 2.7 cc. of Tyrode's solution with no serum. The tissue inoculum of each flask was approximately 0.02 gm. of finely minced tissue from mouse embryos removed during the final week ante partum and from chick embryos incubated for 9 or 10 days. After inoculation with 0.3 cc. of virus suspension the flasks were stoppered with cotton, sealed with tinfoil and incubated at 37.5° C. for 4 or 5 days. Inoculations were made in quadruplicate in each medium. Cultures were examined for bacterial contamination routinely by direct smear or subculture.

For studying cultivation of the virus in the presence of antiviral serum media were prepared as in A except that immune serum was substituted for normal rabbit serum. The difficulty of obtaining convalescent serum of sufficiently high neutralizing power for the encephalitis virus necessitated the preparation of immune serums in animals. This was accomplished by injecting rabbits with the Daily strain intramuscularly three times at weekly intervals with 1 cc. of supernatant fluid from freshly prepared 10 per cent suspensions of brains of mice dying from encephalitis. Protection tests were performed, as previously described,[7] to determine the neutralizing capacity of the rabbit immune serums. Mice inoculated intracerebrally with 0.25 cc. of serum-virus mixtures containing 10^{-3}, 10^{-4} and 10^{-5} dilutions of virus were protected. Under these conditions of immunization each of four rabbits responded with serums of this titer.

In addition to growth by *in vitro* methods the virus was cultivated in the fertilized hen's egg according to the method of Woodruff and Goodpasture. In later experiments artificial air sacs were produced by the technique of Burnet and Galloway.[8] Inoculations were made by placing two drops of virus suspension on the chorio-allantoic membrane. Undiluted flask culture material was used for the initial inoculum and subsequently 10 per cent suspensions of infected chorio-allantoic membranes served for serial passage of the virus. Usually six or more eggs were inoculated for each passage. Eggs were incubated at 38° C. and were observed twice daily. They were opened for final examination on the 2nd to the 10th day after inoculation.

Demonstration of Virus in Culture

The presence of virus in culture media was determined by intracerebral inoculation of mice under ether anesthesia. Tissue, suspended in fluid from the same culture flasks, was ground aseptically without abrasive. After centrifugation for 5 minutes at 2500 r.p.m. the supernatant fluid was injected undiluted or, when titrations were made, diluted with Tyrode's solution to the proper concentration. The C-57 strain of black mice was used except in rare instances when white mice from local stocks were injected. To determine the presence of virus in the inoculated eggs the supernatant fluid from a 10 per cent suspension of ground chorio-allantoic membrane was employed. In testing for virus in a given culture or egg passage generally three, occasionally four or more, mice were inoculated.

The clinical and pathological changes observed in mice infected with virus cultivated in eggs and in the various types of media were identical with those following injection of mouse passage virus.[9]

Experimental

Cultivation in Vitro

As may be seen from the data recorded in Table I, three strains of the virus of St. Louis encephalitis have been grown in each of the four media described. In medium A, containing minced mouse embryo tissue, normal rabbit serum and Tyrode's solution, the Daily strain of virus was cultivated through 26 transplants over a period of 4 months, while the Barnes strain was carried through 25 transplants and the Webster No. 3 strain through 8.

Since growth of the virus was not recognizable visibly in any of the media it was necessary to determine the presence of virus by injecting the cultures into susceptible animals. Equal quantities of material from the quadruplicate culture flasks were pooled and injected undiluted into three or more mice for routine testing. The infectivity of successive generations of subcultures was determined at frequent intervals. Culture material from all three strains was infectious for mice when the experiments were discontinued.

In B, mouse embryo medium containing no serum, the cultivation of virus was less successful. The Webster No. 3 strain failed to survive the first few transplants on three different occasions, and the Barnes strain was lost between the 11th and 14th serial transplants.

TABLE I

Cultivation of St. Louis Encephalitis Virus in Vitro

Tissue cultures		Source of virus	Culture generations in source medium	Cultures in test medium			Total generations in culture	Calculated dilution of original virus in last virulent culture	
Medium	Virus strain			Number of generations	Generations fatal for mice *	Generations not fatal for mice		Since inoculated in test medium	Since isolation from mice
A Mouse embryo in rabbit serum and Tyrode's solution	Barnes	Mouse brain	–	25	6th, 10th, 12th, 15th, 17th, 18th, 19th, 21st, 25th		25	10^{-26}	10^{-26}
	Daily	Mouse brain	–	26	6th, 12th, 15th, 17th, 18th, 20th, 22nd, 23rd, 24th, 26th		26	10^{-27}	10^{-27}
	Webster	Mouse brain	–	8	1st, 3rd, 5th, 8th		8	10^{-9}	10^{-9}
B Mouse embryo in Tyrode's solution	Barnes	Culture in medium A	10	14	2nd, 5th, 7th, 8th, 9th, 11th	14th	24	10^{-12}	10^{-22}
	Daily	Culture in medium A	9	15	5th, 7th, 8th, 12th, 15th	10th	24	10^{-16}	10^{-25}
	Webster	Culture in medium A	5	5		5th	10		
		Mouse brain	–	5		1st, 3rd, 5th	5		
		Culture in medium A	6	3	1st	3rd	9	10^{-2}	10^{-8}
C Chick embryo in rabbit serum and Tyrode's solution	Barnes	Culture in medium D	5	4	2nd, 4th		9	10^{-5}	10^{-10}
		Culture in medium A	4	14	6th, 11th	5th, 13th, 14th	18	10^{-12}	10^{-16}
	Daily	Culture in medium A	4	20	5th, 11th, 13th, 14th, 16th, 18th, 20th		24	10^{-21}	10^{-25}
	Webster	Mouse brain	–	8	1st, 3rd, 5th, 8th		8	10^{-9}	10^{-9}
D Chick embryo in Tyrode's solution	Barnes	Culture in medium C	11	9	3rd, 7th, 9th†		20	10^{-10}	10^{-21}
	Daily	Culture in medium C	11	9	3rd, 5th, 7th, 9th		20	10^{-10}	10^{-21}
	Webster	Mouse brain	–	8	1st, 3rd, 5th, 8th		8	10^{-9}	10^{-9}

* Three mice were usually inoculated, occasionally four or more, with each culture. Except where noted all mice died in from 4 to 7 days with typical convulsions.

† One mouse escaped, one survived, one died with convulsions.

However, the Daily strain was still infectious for mice in the 15th generation when the experiment was discontinued.

Cultures of the Daily strain were maintained through 20 generations in medium C containing minced chick embryo, Tyrode's solution and normal rabbit serum. In the same medium cultures of the Webster strain were virulent through 8 generations, beyond which point further cultivation was not attempted. The Barnes strain was grown in one experiment through 11 successive transplants. Subcultures were continued to the 14th transfer but the last two passages failed to infect animals. The 5th culture generation of this series failed to infect mice, but infections with material from the 6th were typical. Another series of cultures of this strain was discontinued after the 4th subculture although virus was demonstrated at that time. In D, similar medium without serum, the Daily and Barnes strains were transplanted for 9 generations and the Webster strain through 8 generations. The last culture in each case was lethal for mice.

Cultures in medium A were initiated with infected mouse brain. Most cultures in other media were inoculated with culture virus, and the total number of generations *in vitro* was actually greater than is indicated above. These figures are recorded in Table I.

Titrations to determine the quantity of virus present in the cultures were carried out on the 6th and 22nd generations of the Daily strain and on the 6th culture passage of the Barnes strain.

The Barnes strain cultures, after incubation for 5 days, were diluted 10^{-1}, 10^{-2}, 10^{-4} and 10^{-6} and then injected into four groups of four mice each. This culture virus was lethal for all mice receiving 10^{-1} and 10^{-2} dilutions. Higher dilutions had no effect as measured by the presence of clinical symptoms or immunity on subsequent inoculation of mouse passage virus.

Titration of the 6th culture passage of the Daily strain was performed in the identical manner employed with the Barnes titration. The mice receiving dilutions of 10^{-1} and 10^{-2} died on the 5th and 6th days after inoculation. None of the animals injected with the 10^{-4} dilution gave evidence of infection, but two of the group receiving the highest dilution (10^{-6}) died after a longer incubation period. When the six survivors were tested at a later date by intracerebral injection of mouse passage virus two were found to be immune.

The 22nd generation of the Daily strain was titrated at various time intervals (1, 2, 3, 4, 6, 7, 10 and 17 days after inoculation of the flasks) to determine the effect of incubation time on the potency of the virus. Samples tested from the 1st to the 10th day regularly killed mice at a dilution of 10^{-2}, and in some tests (1, 4, 7 and 10 days) killed irregularly at a dilution of 10^{-3}. There was a distinct decrease in the amount of active virus present after 17 days as evidenced by death of only two of three mice receiving undiluted culture and of only one of three injected with the 10^{-1} dilution. Another set of cultures of the same strain was virulent for mice after incubation at 37° C. for 5 days, but not after 29 days.

Titrations determining the amount of virus in inoculated culture media of this type indicate that a 10^{-2} dilution is uniformly lethal for mice and that higher dilutions irregularly kill mice or immunize them to subsequent infection by inoculation. Further, although the titer of culture virus was much lower than that of virus in the brain of passage animals, the data show that there was no significant decrease in potency during the 16 culture passages intervening between two sets of titrations of one (Daily) strain.

Cultures with Immune Serum

An attempt was made to cultivate St. Louis encephalitis virus on mouse embryo tissue in the presence of rabbit serum containing neutralizing antibodies against the virus. Control cultures were grown in mouse embryo medium with normal rabbit serum, test cultures in the same medium with immune serum. Fresh mouse passage virus (Daily strain) was used for inoculum. Cultures were transplanted at intervals of 4 or 5 days into the same kinds of medium. In addition, each culture in immune serum was subcultured in medium containing normal serum by transplanting whole culture material in some cases, in others tissue fragments which had been centrifugated and resuspended in Tyrode's solution in order to remove most of the immune serum.

Culture material from both control and test cultures was washed to remove the serum before inoculation into mice. All mice injected with material from control cultures in normal serum medium died. None were affected by material from immune serum cultures or from 1st, 2nd or 3rd subcultures of these in control medium, and all the survivors succumbed when subsequently inoculated with mouse

passage virus. Thus the virus either failed to survive in the presence of immune serum or was modified to such an extent that it failed to infect or immunize mice. The experiment was discontinued after 4 culture passages.

Cultivation in Vivo

Two strains of St. Louis encephalitis virus were inoculated in series in 13 to 16 day old chick embryos. The embryos were often alive and active 5 or 6 days after infection with virus and occasionally hatched normally. However, the majority died on the 4th or 5th day after injection.

Three attempts were made to propagate the Barnes strain in serial passage in eggs. In each case the initial inoculum consisted of a suspension of ground tissue from a flask culture. One experiment was terminated early by bacterial contamination. In another the original inoculum proved to be non-infectious for mice but passage was continued for a number of generations as a control series. Mice inoculated with material from embryos and membranes in this series remained alive and apparently unaffected by the foreign tissue. The third experiment was continued in duplicate series through 9 egg passages.

Four series of egg passages were begun with the Daily strain. In two earlier series in which younger embryos were used the virus was lost by the 3rd passage. Another series was discontinued after a few passages although the virus was still potent. The fourth series was continued in two parallel experiments through 10 passages.

As indicated in Table II, the Barnes strain of the virus was of maximum infectivity for embryos and mice in each of the first 7 passages. It was recovered from the brain as well as the membranes of the 4th and 6th passage embryos. Mice injected with material from 8th passage embryos died apparently of bacterial infection. There were no lesions in the chorio-allantoic membranes of the 9th passage embryos, and mice injected with tissue from them were not infected.

The Daily strain was recovered from embryos in each of 10 serial passages of the virus by inoculation of mice with chorio-allantoic tissue. Mice died with typical encephalitis also after inoculation with brain, liver and spleen of an embryo in the 7th passage, and with liver from an embryo in the 9th passage.

TABLE II

Growth of St. Louis Encephalitis Virus in Developing Chick Embryos

Virus strain	Egg passage †	Embryos used for egg passage and mouse inoculation				Results of mouse inoculation *	
		Age in days when inoculated	Days of incubation after inoculation	Condition of embryos	Character of lesions in chorio-allantoic membranes	With chorio-allantoic membranes	With other embryo tissues
Barnes	1	14	3	1 alive	Proliferative	5, 5, 5	
			7	1 dead	Large, necrotic center	5, 7, 7	
			8	1 hatched paralyzed			
	2	15	6	1 alive	Large, necrotic center	6, 6, 7	
			2	1 dead	Cloudy patches	6, 6, 6	
	3	15	7	1 dead	Large, necrotic center	6, 6, 6	
	4	12	10	2 dead	" " "	6, 6, 7	7, 7, 8 (brain)
	5	14–16	4–6	3 dead 1 hatched	" " "		
	6	14	3	2 dead	" " "	4, 5, 7	
			6	1 alive	" proliferative	6, 6, 6	6, 6, 6 (brain)
	7	14–15	4–6	2 dead	" necrotic center	6, 6, 6	
	8	15	6	2 dead	" necrotic	2, 2, 2‡	
			5	1 alive	" "		
	9	15	4	1 alive	" "	s, s, s	s, s, s (liver)

Daily	1	15	4	1 alive	Proliferative	5, 6, 6	
	2	13–14	5	1 dead 1 alive	"	6, 6, 6	
	3	13	5	1 alive	"	1, 7, 7	
	4	14	5	1 dead	"	6, 6, 8	
	5	12–16	7–9	2 dead	Large, necrotic center	7, 7, 8	
	6	13–15	6	2 dead	Proliferative	6, 6, 6	
	7	14	4–6	2 alive	Large, necrotic center	6, 6, 7	6, 6, 6 (liver and spleen) 6, 6, 6 (brain)
	8	14	4	1 dead	" " "	1, 5, 5	
		14–15	4	2 alive	" " "	6, 6, 6	
	9	16	4–5	2 alive	" " "	4, 6, 6, 8	6, 6, 7 (liver)
	10	12–15	3–4	2 alive	" " "	5, 5, 6, 7, 7, 8	

* Three or more mice were inoculated intracerebrally with each tissue suspension. Figures represent dates of death. s = survived.
† Six or more eggs inoculated for each passage.
‡ These mice apparently died of bacterial infection.

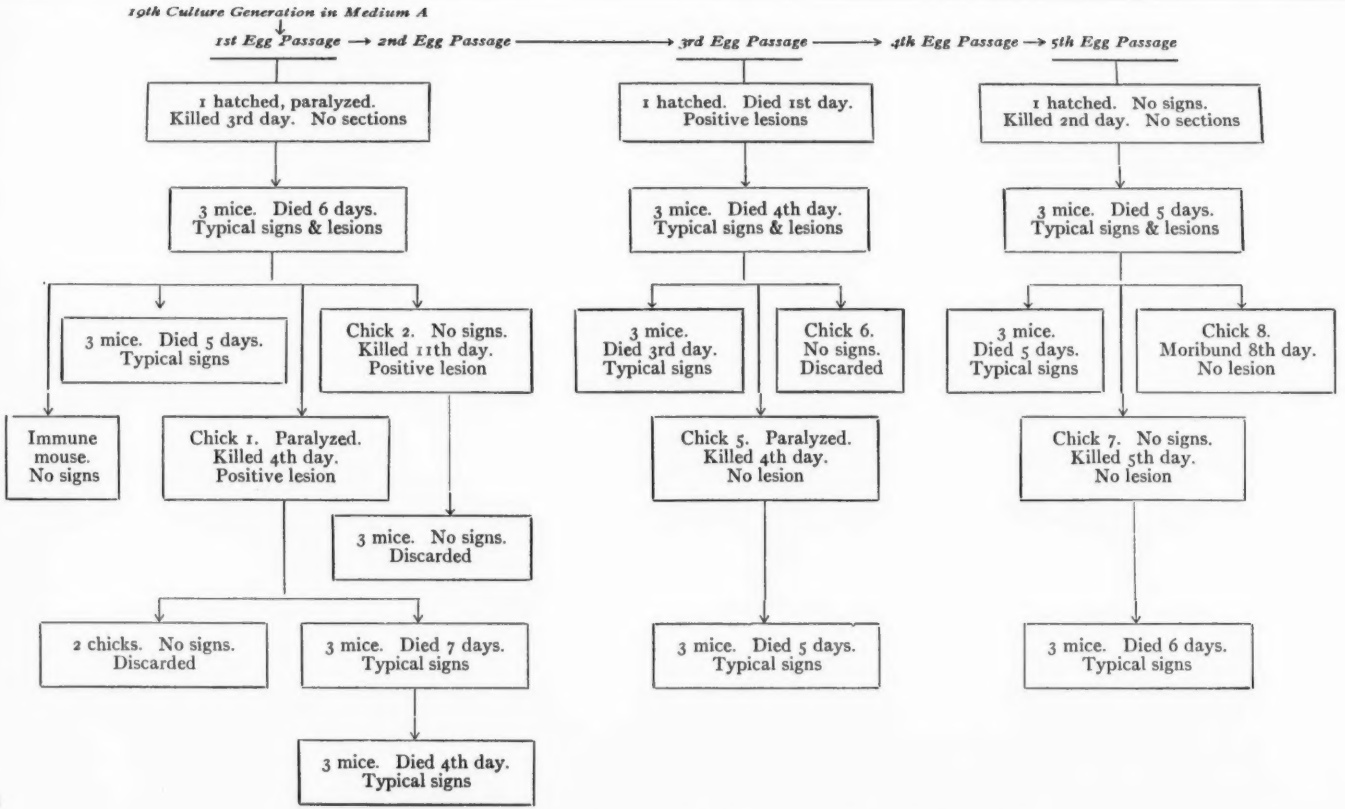
Changes Observed in Infected Chick Embryos

The pathogenicity of St. Louis encephalitis virus for the chick embryo was evidenced by the lesions of the chorio-allantoic membranes and by frequent death of the embryos. The lesion of the chorio-allantoic membrane appeared first as an opacity, then a thickening, and finally, if the embryo lived long enough, a definitely necrotic patch at the site of inoculation. Often the gray patch of proliferation spread out beyond the triangular area under the window, but was of maximal intensity at that site. Microscopically a proliferation of all three germ layers was observed. Early lesions consisted mainly of proliferation of the ectoderm, but as time went on the other two layers became involved. The ectoderm became thickened to a depth of six to eight cells, with the formation of epithelial pearls. Proliferation of the endoderm was much less pronounced. In the mesoderm an increase of fibroblasts and an invasion of epithelial cells were seen, and often large mononuclear cells were grouped around vessels. In some cases there was an exudate on the ectodermal surface, composed of polymorphonuclear and mononuclear cells with pyknotic nuclei and many erythrocytes. When an exudate existed on the ectoderm there was generally a sprinkling of polymorphonuclear cells and erythrocytes throughout the mesoderm. The vascularity of the ectoderm was greatly increased. No cell inclusions were seen and no pathological changes were found in brains of infected embryos, including those from which virus was recovered by inoculation of mice with brain tissue, although, as described below, lesions were encountered in the brain of a chick that hatched after inoculation.

Infection of Young Chicks

One egg in each of the 1st, 2nd, 3rd, 5th and 6th passages of the Barnes strain was incubated until it hatched. The chicks from the 2nd and 6th passages died while hatching and were discarded. Brain tissue from the others was used for inoculation of mice and young chicks in the experiments recorded in Text-figure 1. The chick hatched from a 1st passage egg, paralyzed in both legs, was killed after 3 days and a 10 per cent suspension of its brain was inoculated intracerebrally into mice. These developed convulsions and were killed when moribund 6 days after inoculation. The micro-

19th Culture Generation in Medium A


19th Culture Generation in Medium A
1st Egg Passage
2nd Egg Passage
3rd Egg Passage
4th Egg Passage
5th Egg Passage
1 hatched, paralyzed. Killed 3rd day. No sections
1 hatched. Died 1st day. Positive lesions
1 hatched. No signs. Killed 2nd day. No sections
3 mice. Died 6 days. Typical signs & lesions
3 mice. Died 4th day. Typical signs & lesions
3 mice. Died 5 days. Typical signs & lesions
3 mice. Died 5 days. Typical signs
Chick 2. No signs. Killed 11th day. Positive lesion
3 mice. Died 3rd day. Typical signs
Chick 6. No signs. Discarded
3 mice. Died 5 days. Typical signs
Chick 8. Moribund 8th day. No lesion
Immune mouse. No signs
Chick 1. Paralyzed. Killed 4th day. Positive lesion
Chick 5. Paralyzed. Killed 4th day. No lesion
Chick 7. No signs. Killed 5th day. No lesion
3 mice. No signs. Discarded
2 chicks. No signs. Discarded
3 mice. Died 7 days. Typical signs
3 mice. Died 5 days. Typical signs
3 mice. Died 6 days. Typical signs
3 mice. Died 4th day. Typical signs

Text-figure 1. Passage of St. Louis encephalitis virus (Barnes strain) through young chicks.

scopic lesions observed in the brain were typical of those caused by the virus. Brain emulsion from these mice inoculated into three normal mice induced typical convulsions and death on the 4th and 5th days, while a similar inoculation in a mouse recovered from a previous infection with the Daily strain was without effect. The same mouse brain suspension was also inoculated intracerebrally into two young normal chicks, 4 to 6 days old. One of these remained well and the virus was not recovered when it was killed 10 days later, despite the fact that lesions were found in sections of the brain. The other became partially paralyzed and was sacrificed 4 days after inoculation. Brain suspension from it induced no symptoms in two young chicks but caused death in mice within 7 days. Virus recovered from them killed a second set of mice on the 3rd and 4th days. This experiment was not continued further.

The chick obtained from the 3rd egg passage died on the day it emerged from the shell. The fifth, apparently normal, was sacrificed for study on the 3rd day. Brains from both these chicks were used for passage of the virus through mice and young chicks As indicated in Text-figure 1 the results were similar to those described above. In each case the virus was recovered from two sets of mice, inoculated serially, and from chicks.

Thus, the St. Louis virus, recovered from the brain of each of three newly hatched chicks, which had been inoculated on the chorio-allantoic membrane 5 to 8 days before hatching, was passed in series through mice and young chicks. Microscopic pathological changes were seen in the brain of a hatched chick from the 3rd egg passage, and in two normal chicks that were inoculated, after hatching, with mouse brain infected with 1st egg passage virus. They consisted of extensive perivascular cuffing with mononuclear cells and an occasional small glial nodule but no meningeal reaction. One chick in each of the other two series exhibited clinical signs of infection but no pathological changes were observed in sections. Further chick to chick passage in the first series produced no signs or lesions, although the virus was present in the passage material as demonstrated by mouse inoculation. Mice inoculated with brain emulsions from infected chicks exhibited clinical and pathological pictures characteristic of St. Louis encephalitis.

The occurrence of paralysis and pathological changes in the brains of certain chicks indicate a potential susceptibility of young chicks

to the virus. Also, there is a suggestion that chicks may carry the virus without manifestation of infection. It is interesting in this connection that virus was not recovered from Chick 2, sections of which showed encephalitic changes.

Discussion

Three strains of the virus of St. Louis encephalitis have been cultivated *in vitro* in media composed of minced mouse embryo tissue suspended in Tyrode's solution, either with or without normal rabbit serum, but the virus failed to survive when immune serum was present. Two different strains of virus were infectious for mice in the 25th and 26th subcultures *in vitro* when observations were discontinued. These final generations represented an approximate dilution of 10^{-26} and 10^{-27}, respectively, of the original infected mouse brain. Since the approximate final dilution in cultures was far beyond the end point of infectivity of the original mouse brain,[10] and since virus kept under conditions not favorable to growth becomes inactive within a few hours,[11] propagation of the virus *in vitro* undoubtedly occurred.

The experiments reported here agree in general with the observation of Syverton and Berry [3] that the infectious titer of culture virus is in the neighborhood of 10^{-2}. Although this titer is definitely lower than that occurring in routine mouse passage virus it was maintained over a period of at least 22 culture generations. Cultivation of the virus *in vitro* does not appear to alter its characteristic effects on mice, as it induced the usual clinical signs and pathological changes after an incubation period typical of mouse passage virus.

The virus was successfully cultivated also in similar media containing chick embryo tissue and in developing chick embryos. It induced changes in the chorio-allantoic membranes and remained virulent for mice through 10 serial transplants in the fertile egg. Virus was recovered from the brain, liver and spleen, as well as from the chorio-allantoic membranes of infected chick embryos and from the brains of chicks that hatched after being infected. Since the 10th egg passage material represented a dilution of the original inoculum of about 10^{-32} it may be assumed that the virus actively multiplied in the chick embryo.

Although mature chickens are not affected by the virus [10] very

young chicks were found somewhat susceptible. Half of the small number used showed clinical or pathological changes, or both, and virus was recovered in all but 1 of the passages from chick to mice.

The modified neurotropism of the St. Louis virus, demonstrated by Webster and Clow [12] in mouse experiments, is further exemplified in the present observations. Histological changes in all three germ layers of the chorio-allantoic membrane, together with the isolation of virus from the liver and spleen of developing chick embryos, confirm and extend the observations of Webster and Clow concerning the viscerotropic action of the virus.

Summary

1. Three strains of the virus of St. Louis encephalitis have been successfully cultivated *in vitro* in the presence of living cells.

2. The media employed were minced mouse or chick embryo tissue suspended in Tyrode's solution, either with or without rabbit serum.

3. Media containing normal rabbit serum supported the growth of virus better than media without serum. When rabbit serum possessing neutralizing antibodies against the virus was employed in cultures the infectious agent was not recovered.

4. Two strains of the virus were propagated in serial passage in the developing chick by inoculation on the chorio-allantoic membrane. The virus was recovered from the brain, liver and spleen, as well as from the chorio-allantoic membrane of passage chick embryos. Histopathological changes occurred in all three germ layers of the embryonic membrane.

5. Young chicks are to some extent susceptible to the virus.

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FIBROSIS OF THE BONE MARROW (MYELOFIBROSIS) ASSOCIATED WITH A LEUKEMOID BLOOD PICTURE *

Report of Two Cases

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In 1879 Heuck [1] described a case of leukemia in which the bone marrow at autopsy was found encroached upon by a network of fibrous tissue and by fine bony trabeculations. This condition was later classified as osteosclerosis by Kaufmann in his textbook of special pathological anatomy. From time to time other cases of osteosclerosis have been reported in the German literature. The subject was well reviewed by Oesterlin [2] in 1924, by Mavros [3] in 1931, and by Anagnostu [4] in 1933. More recently similar cases have been reported in this country by Stephens and Bredeck [5] and Chapman.[6]

Following the early publications of Heuck,[1] von Jaksch,[7] Askanazy,[8] and Nauwerck and Moritz,[9] two types of osteosclerosis were recognized: the so-called marble bone or Albers-Schönberg [10] disease, and myelosclerosis or myelofibrosis. In the former type the essential lesion is one primarily of excessive bone formation, with gradual thickening of the cortex and encroachment on the medullary cavity. The condition occurs usually in childhood, persists throughout life into adulthood and terminates with a myelophthisic anemia. The latter type of the disease occurs most often after the second decade, and a leukemoid blood picture with enlargement of the spleen and severe anemia are the outstanding clinical findings. The pathological process in the medullary cavities consists of fibrosis with some bone formation.

The case reports which follow are, we believe, of the latter type, presenting leukemoid blood pictures and fibrosis of the bone marrow.

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Case Reports

Case 1.* A. A. W., a white American housewife, aged 40 years, consulted her physician on July 14, 1923, because of pains in the left arm and the legs, and the appearance of purpuric spots in the skin. She stated that she had been "pale and anemic" since childhood and had received injections of iron for this condition. However, an examination of her blood by a physician on Dec. 4, 1919, had shown that the red blood cells were 4,280,000 in number, with a hemoglobin reading of 88 per cent. On Feb. 14, 1922, examination of the blood had given approximately the same results. Six months prior to visiting her physician an operation had been performed for suspension of the uterus. Bleeding was not unduly notable at that time. At the time of operation the hemoglobin was reported to be 85 per cent. In May, 1923, a sharp, stabbing pain occurred in the left arm. More recently a similar pain developed in the knees and the upper portion of the sternum. During the month of May the appearance of purpuric spots was noticed in the skin, there was a tendency to bleed from the nose, and the menstrual flow was prolonged. At times there had been bleeding from the gums, but blood had not been seen in the urine or feces.

Physical Examination: In July, 1923, extreme pallor of the skin and mucous membranes and the presence of many small purpuric spots in the skin was noted. There was no evidence of organic heart disease or infection in the ears, the throat or about the teeth to account for her condition. There was no demonstrable enlargement of the spleen, liver or superficial lymph nodes.

Laboratory Data: Examination of the blood showed: red blood cells 2,080,000 per cmm., hemoglobin 43 per cent, and white blood cells 4200 per cmm. The differential count was: neutrophiles 22 per cent, myelocytes 43 per cent, unclassified cells 16 per cent, lymphocytes 15 per cent, and monocytes 4 per cent. The 16 per cent unclassified cells were thought probably to be myelocytes. An occasional normoblast was seen. The blood platelets were obviously reduced in the blood smear. A specimen of blood clotted in 1½ minutes but failed to retract. Bleeding from a small puncture of the ear continued after 7 minutes.

Course of Illness: During the following months, from July to December, the patient's hemorrhagic symptoms were ameliorated by transfusions of whole blood, given at intervals of every 3 to 4 weeks. She continued, however, to have a persistent leukopenia and an anemia, and at times the platelets were extremely scant in the blood smear. The spleen was not found enlarged by palpation, nor did it cast an abnormally large radiographic shadow until November.

In December, 1923, splenectomy was performed and the operation was followed by a rise in the platelet count from approximately 10,000 to 100,000 per cmm. Following operation the gums bled slightly for a few days and then all bleeding stopped. The patient showed general symptomatic improvement until March, 1924, when the purpuric spots, joint pains and bleeding of gums returned.

On March 25th examination of the blood showed: red blood cells 2,400,000, hemoglobin 36 per cent (Sahli), color index 0.75, and white blood cells 25,900. The differential count was: neutrophiles 10 per cent, lymphocytes 31 per cent, monocytes 5 per cent, and myelocytes 54 per cent. Her condition became rapidly worse and she died on March 29, 1924.

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* The authors wish to thank Drs. Fletcher Taylor, George Evans and Ernest Falconer for permission to use the clinical data on this case.

Autopsy Abstract *

The body appeared well developed and fairly well nourished. There was extreme pallor of the skin and mucous membranes. In the skin over the chest, abdomen, arms and legs there were numerous areas of petechial hemorrhage measuring up to 3 mm. in diameter. The gums were spongy and soft with bleeding surfaces. The neck showed no palpable lymph nodes or enlargement of the thyroid. The abdomen was considerably distended. Along the left costal margin was a long scar from the surgical incision for splenectomy. Between the umbilicus and the symphysis pubis was found another linear scar of an old operation. There was a moderate amount of fat in the abdominal wall. On opening the peritoneal cavity a few, dense fibrous adhesions were seen between the omentum and abdominal wall, beneath the diaphragm, and about the stomach in the region from which the spleen was removed.

Heart: The heart weighed 270 gm. There were three small areas of petechial hemorrhage beneath the epicardium and a few beneath the endocardium of the right auricle.

Lungs: The lungs showed nothing noteworthy except slight emphysema.

Liver: The liver weighed 2070 gm. Fairly fresh adhesions were found over the superior surface of both lobes and rather dense adhesions involved the region adjacent to the stomach. The tissue was extremely pale and somewhat flabby. The cut surface was smooth and glistening and showed several, minute, circumscribed whitish areas. The lymph nodes at the hilus of the liver were greatly enlarged, soft and hyperemic.

Gall-bladder: Normal.

Pancreas: Normal.

Spleen: The spleen was missing on account of operative removal.

Stomach: The stomach was distended and filled with a watery, slightly hemorrhagic fluid.

Ileum: There was a diffuse hemorrhage beneath the mucosa and serosa in the wall of the ileum.

Kidneys: The combined weight of the kidneys was 320 gm. The capsules stripped readily, leaving an exceedingly pale surface which showed the remains of fetal lobulation. Numerous petechial hemor-

* Autopsy No. A–24–87.

rhages were found on the surfaces. On cut section nothing of note was found except marked pallor.

Uterus and Adnexa: The uterus was approximately normal in size. There were some old, healed, irregular lacerations of the cervix. The appendix, right Fallopian tube and ovary were absent as a result of surgical removal. The left ovary contained three large cystic areas filled with clotted blood.

Bone Marrow: The marrow of the ribs and of the femur was of a pale grayish color. There was a large amount of cancellous bone and fibrous tissue which extended throughout the medullary portions of the bones.

Histological Examination

Examination of the thyroid, lungs, heart, kidneys, pancreas, intestines and adrenals was consistent with the characteristics noted by gross inspection.

Liver: A section of liver showed scattered, miliary-like foci of cells which were chiefly of a large mononuclear type. The nuclei were large and contained an abundance of chromatic material enclosed in a definite nuclear membrane. The nuclei were surrounded by varying amounts of acidophilic cytoplasm. Comparatively large numbers of a similar type of cell were to be seen in the sinusoids. No foci of erythropoiesis were seen. In the liver cells about the periphery of the lobules a deposition of golden brown pigment was found.

Lymph Nodes: A section of a retroperitoneal lymph node showed definite alteration of its architecture. No germinal centers were seen. The tissue was moderately cellular and composed mostly of small mononuclear cells resembling lymphocytes. Interspersed among these, however, were seen large mononuclear cells, each containing a large round nucleus. The nuclear membrane was well defined and surrounded a sparse amount of chromatic material. The cytoplasm about these nuclei varied considerably in amount in individual cells stained with eosin. The type of cell seemed to be of myeloid origin. An occasional large multinucleated cell which resembled the megakaryocytes of the bone marrow was also seen. The sinusoids were greatly dilated and contained large numbers of mononuclear cells which presumably were myelocytes. In the sinusoids were to be seen an occasional megakaryocyte. There was no evidence of erythropoiesis.

Bone Marrow: A specimen of bone marrow removed from the femur showed tissue made up of blood formative cells and fibrous tissue. In some areas were myelocytes and myeloblasts forming compact hyperplastic foci of leukopoiesis. Elsewhere the cells were loosely arranged and scattered. Only occasional small foci of erythropoiesis were noted. A loose connective tissue extended throughout the section of bone marrow between small spicules of bone. In this connective tissue were large numbers of fibroblasts. This tissue was moderately vascular. No megakaryocytes were seen.

A specimen of bone marrow removed from the rib showed tissue that was, in general, more cellular than the femoral marrow; however, the connective tissue reaction was greater and in some places completely replaced the marrow elements. Proximal to the cancellous portion of the bone, in some areas, dense strands of wavy connective tissue fibrils were to be seen. In such areas there was but scant evidence of hematopoiesis.

Spleen (Surgical Specimen): The architecture was loose in structure and supported a moderately cellular pulp. Numerous cells of the myeloid series were to be seen and suggested metaplasia consistent with the diagnosis of leukemia. In the sinusoids large mononuclear cells resembling myeloblasts were to be seen. The malpighian bodies of the spleen appeared to be present in normal size and numbers. There was no evidence of erythropoiesis.

CASE 2. A. M., an American male, aged 34 years, married, was admitted to the Mount Zion Hospital on April 25, 1935, complaining of general malaise, weakness, fever and sweating of about 2 weeks duration. He first came to the Clinic in May, 1932, at which time a diagnosis of chronic myeloid leukemia was made, and since that time he had been under observation at frequent intervals. Because of a high white cell count, irradiation by roentgen ray had been administered between May and October, 1932, and he had received a total of 800 R over the spleen and abdomen. In 1933, during the course of the year he had received four treatments totalling 400 R. In 1934 he had received a total of 630 R over the abdomen. During 1935, from February to the time of death, 5 months later, he received 500 R over the abdomen and extremities.

Two weeks prior to entry in the hospital he began having afternoon chills followed by profuse diaphoresis. There was no pain but at times he complained of generalized "body ache."

Physical Examination: On entry to the hospital the patient was well developed but undernourished and appeared quite ill. The temperature was 39.5° C. (102.9° F.); the pulse rate was 124; and the respiratory rate 28. The mucous membranes were pale and the skin moist. The ears, teeth, nose, throat and lymph nodes presented no remarkable abnormalities. There was no evidence of cardiac disease. The blood pressure was 120 systolic and 70 diastolic. Extreme

tenderness was elicited on pressure over the sternum. The edge of the spleen was felt 4 cm. below the left costal margin, and the edge of the liver 3 to 4 cm. below the right costal margin.

Laboratory Data: The laboratory findings, with the exception of the blood counts which are recorded in Table I, were essentially negative. A sample of venous blood obtained on May 27, 1935, was reported as yielding no growth of organisms.

Clinical Course and Treatment: The patient did not show much change in his condition until May 18, 1935, when he suddenly became extremely weak and dyspneic. On this date he was given a transfusion of 500 cc. of whole blood. The following day the patient complained of impaired vision in the right eye. On ophthalmoscopic examination numerous small hemorrhages were seen in each fundus. On May 23, 1935, he received another transfusion of 500 cc. of whole blood. Despite this treatment the course of the patient's illness was progressively downhill. On May 31, coarse and crepitant râles became audible in both lungs, and on this date the patient expired.

Autopsy Abstract

The body was that of a white adult male appearing to be about 35 years of age, poorly nourished but normally developed. Extreme pallor of the skin and mucous membranes was present. There was no enlargement of lymph nodes in the neck or popliteal spaces, but a few enlarged lymph nodes were found in the axillae. The liver was palpable in the right upper quadrant. The external genitalia were normal, as were the extremities. The usual midline incision showed a normal peritoneal cavity. The liver edge extended two finger-breadths below the costal margin, and the spleen was greatly enlarged. The pleural and pericardial cavities appeared normal. No abnormal lymph nodes were found in the mediastinum, mesentery or retroperitoneum.

Heart: The heart was of normal size and shape. There was no evidence of endocarditis.

Lungs: The lower lobes of both lungs were "boggy" and congested because of a bronchopneumonia.

Gastro-intestinal Tract: The esophagus was normal. There were some minute discolored foci in the mucosa of the stomach, suggestive of ulcerations. The small and large intestines were normal.

Trachea: There were no gross changes in the true and false vocal cords.

Liver: The liver was about one and a half times normal size, soft and uniform in consistence, and a light brown.

Spleen: The spleen was about twice normal size, firm in consist-

TABLE I

Data Showing the Progressive Development of Severe Anemia, Thrombocytopenia, and the High Percentage of Immature Leukocytes in the Differential Formulas Reported in Case 2

Date	Hemoglobin (Sahli)	Red blood cells in millions per cmm.	Platelets per cmm.	White blood cells per cmm.	Polymorphonuclears		Polymorphonuclear eosinophiles	Polymorphonuclear basophiles	Lymphocytes	Monocytes	Myelocytes	Myeloblasts	Normoblasts per 100 W.B.C.	Treatment
					Filamentous	Non-filamentous								
	%													
	85													
5/11/32	11.9 gm.	4.95		68,500	16	51	4	1	8	1	18	1	2	X-ray started
10/27/32	90			11,500	47	18	4	3	23	4	1			To date received 800 R
	93													
1/13/33	14.4 gm.	5.44	320,000	16,800	55	12	4	6	17	5	1			400 R
12/12/33				17,750	50	19	2	6	11	6	6			
	86													
4/3/34	13.4 gm.			26,600	37	25	4	4	16	6	8			630 R
	88													
12/29/34	13.6 gm.			10,400	71	3	1	5	11	5	4			
	54													
4/19/35	8.3 gm.				12	39	2	2	4	4	31	6		1% ? megakaryocyte
	48													
4/26/35	7.6 gm.	2.57	100,000	26,250	7	45	4	1	15	1	12	15	3	1% ferrata cell
	28													
5/21/35	4.4 gm.	1.61	135,000		2	11			38	3	1	45	3	Entered hospital
	33													
5/27/35	5.2 gm.	1.97		7,500	0	5			24	3	6	62	7	Transfusions 2 X-ray 500 R

ence and a uniform maroon red. On cut section the normal splenic parenchyma appeared to be replaced by a reddish cellular material. The malpighian corpuscles and the fibrous trabeculations were not evident.

Pancreas: The pancreas appeared normal.

Kidneys: The kidneys were slightly enlarged and equal in size. The capsule stripped with ease, showing a normal smooth surface. On cut section the cortex and medulla were found to be sharply demarcated. The pyramids stood out and there was no evidence of leukemic involvement of the parenchyma. The adrenals, ureters, bladder and prostate were apparently normal.

Bone Marrow: The marrow of the femur was firm, red, and appeared to be hyperplastic.

Histological Examination

Examination of heart, stomach, pancreas, kidneys, prostate, aorta, adrenals and thyroid tissue showed nothing of significance.

Liver: Microscopic examination of the liver showed the presence of a normal architecture. Within the liver cells, especially about the periphery of the lobules, was a deposition of yellowish brown pigment. The Kupffer cells were approximately normal in size, although occasional cells showed considerable hypertrophy. Many of these cells were filled with yellowish brown pigment. The sinusoids showed an unusually large number of cells, the majority of which were large in size, and each contained a large round nucleus. The nuclei for the most part showed a distinct membrane and contained one or more clumps of deeply stained basophilic chromatin. The cytoplasm of these cells varied in amount from a thin rim up to a rather abundant, irregularly shaped mass. The cytoplasm showed faint staining with eosin. Among these cells was an occasional mitotic figure. These were all immature types of cells of the leukopoietic series, and apparently in the myeloblast and promyelocyte stages of development. The adult forms of polymorphonuclear leukocytes were rarely seen. Both the large and small forms of lymphocytes were apparently reduced in number. The red blood cells were comparatively sparse in number; many of these showed considerable anisocytosis and poikilocytosis and seemed to contain a decreased amount of hemoglobin. Normoblasts were exceedingly few in number. An occasional, large mononuclear type of cell, with

an abundant, irregularly shaped cytoplasm was noted. Within the cytoplasm of such cells were seen phagocytozed red blood cells and brown pigment. There were no accumulations around the periportal spaces.

Testes: There were two or three areas of accumulations of immature leukocytes, with an occasional polymorphonuclear.

Spleen: The essential structure was still present but the finer details appeared somewhat disrupted by the presence of numerous unusual cells throughout the pulp. These cells were large, and each contained the large nucleus that often appears in mitotic configuration. Only an occasional adult leukocyte was noted. There was no evidence of erythropoiesis. The malpighian follicles were small and the trabeculae thin. There was no unusual formation of fibrous tissue.

Bone Marrow of Femur: The usual marrow structure was entirely replaced by a cellular fibrous tissue in which were scattered hematopoietic cells. In the more cellular areas, of which there were few, the fibrous tissue was rather loose in structure. The leukopoietic tissue was mainly represented by a large type of cell such as was found in the liver and spleen. This presumably was a myeloblast. Myelocytes and an infrequent small group of normoblasts were present. Mitotic figures were noted frequently throughout, especially in the large undifferentiated cells. No megakaryocytes were to be found.

Costal and Sternal Marrow: There appeared to be a combination of an extensive fibrosis with scattered persisting areas of hematopoiesis in the costal and sternal marrow.

Trachea: A specimen of marrow from a bone in the trachea showed a similar pattern of fibrous tissue but was slightly more cellular.

Discussion

The clinical findings in the 2 cases reported here are similar only in the qualitative aspects of the so-called "leukemoid" blood pictures. The illness of the first patient described was characterized by a hemorrhagic tendency, thrombocytopenia and severe anemia. Enlargement of the spleen occurred late in the course of illness. At the outset a leukopenia occurred and immature forms of leukocytes were found in the blood smear. Prior to death a leukocytosis de-

veloped, and concomitantly there was a definite increase in the number of immature leukocytes. In the second patient the classical findings of myelogenous leukemia were encountered when the patient first sought medical aid. There was splenomegaly and leukocytosis, and myelocytes were present in the blood film. As the course of illness approached termination, myeloblasts appeared in the blood and the patient developed in addition a severe anemia.

On postmortem examination these 2 cases presented a striking similarity in the structural changes of the long bones. This is shown in Figures 1, 2 and 3, where it is to be noted that the medullary cavities showed increased fibrosis and a few spicules of cancellous bone which partially obliterated the hematopoietic tissue.

In the 1st case, substantiation for the diagnosis of leukemia was found elsewhere in the body, as evidenced by the extramedullary proliferation in the spleen, liver and lymph nodes. The pronounced alteration of the lymph nodes was perhaps the most characteristic feature of leukemia. The normal architecture was largely obliterated. Myeloid cells in various stages of maturation were present in the sinusoids and uniformly scattered among the remaining lymphoid cells. Megakaryocytes were occasionally seen. In the 2nd case, extramedullary leukopoiesis was of lesser degree, and there was neither apparent involvement of lymph nodes nor periportal infiltration in the liver. The sinuses of the liver and spleen were, however, filled with cells of a type transitional between myeloblasts and adult forms.

Extramedullary erythrocytopoietic activity has been described in some cases of myelofibrosis. A careful study of the sinusoids of the liver and spleen in our 2 cases, however, failed to reveal any erythrogenetic foci.

In the 1st case reported here the blood picture was compatible with a diagnosis of aleukemic myelosis, whereas in the 2nd case the findings were those typical of myeloid leukemia.

Stephens and Bredeck,[5] in their review of the literature up to 1932, were able to report 19 cases of fibrosis of the bone marrow. All of these cases were consistent with a diagnosis of leukemia. The leukocyte counts varied from as low as 5200 to 80,000 per cmm., and about one-third of these could be designated as aleukemic. Myelocytes and normoblasts were found in the blood smears, and there was enlargement of the spleen in all cases. Since their report a

few other cases with a leukemoid blood picture have been recorded in the literature. Anagnostu [4] described a case of systemic osteosclerosis in a male aged 65 years who had an anemia, a leukocyte count of 3900, and promyelocytes and myelocytes in the blood film. The spleen was moderately enlarged. The combined clinical and postmortem diagnosis was aleukemic myelosis with bone marrow fibrosis. Two cases of osteosclerosis are reported by Chapman [6] in which splenomegaly, anemia and leukopenia occurred, and immature forms of leukocytes were found in the blood film. In these cases biopsy of the sternal marrow showed the presence of fibrosis and established the diagnosis.

There is considerable discussion in the literature as to the exact relation of the leukemoid blood picture to the marrow fibrosis and osteosclerosis. The changes have been thought by some observers to be similar to those occurring in myelophthisic anemia associated with a carcinomatous metastasis to the bone marrow. This conception would presuppose that some initial infection or bone marrow injury had led to the fibrosis, and that the resulting blood changes were secondary to this process. On the other hand, the comparatively large number of cases showing manifest leukemia and a high white count suggests that the fibrosis and bony overgrowth occur secondary to the marrow hyperplasia and may represent the desmoplastic reaction that is characteristic of some cases of carcinoma.

Summary

Two cases of fibrosis of the bone marrow associated with leukemoid blood pictures are presented. In the 1st case the onset of illness was characterized by leukopenia and a hemorrhagic tendency, and later with moderate enlargement of the spleen and a terminal leukocytosis. The course of illness in the 2nd case was typical of leukemia for a period of 3 years prior to the patient's death. In both cases during the postmortem examination there were found in the liver, spleen and lymph nodes changes compatible with a diagnosis of leukemia, but there was partial obliteration of the marrow cavities with fibrous tissue and bony spicules.

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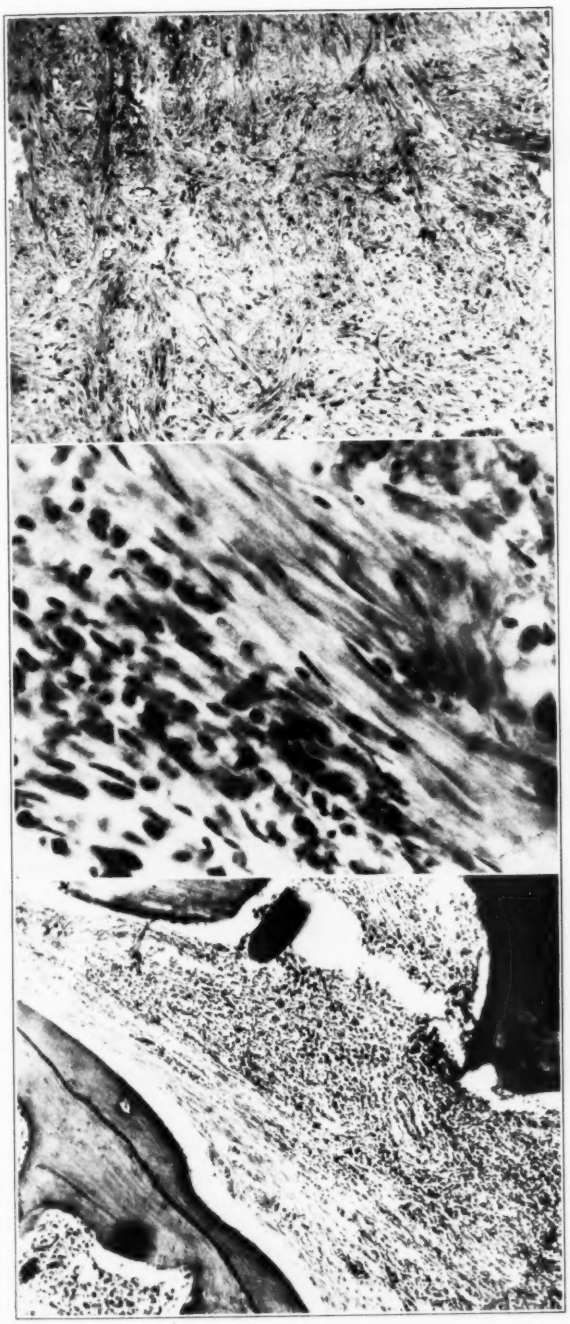
DESCRIPTION OF PLATE

PLATE 59

FIG. 1. Case 1. Showing partial fibrosis of a specimen of femoral bone marrow removed at autopsy. × 110.

FIG. 2. Same as Figure 1, but higher power. × 250.

FIG. 3. Case 2. Showing extreme fibrosis of a specimen of femoral marrow removed at autopsy. × 110.



Mettier and Rusk Fibrosis of Bone Marrow

UNIV. OF MICH.

THE AORTIC COMMISSURAL LESION IN RHEUMATIC FEVER *

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One of the most conspicuous gross lesions in the rheumatic heart is the commissural agglutination of the aortic cusps. In its characteristic form it consists of an adhesion of adjoining sigmoid cusps which extends for a variable distance toward the center of each involved leaflet but preserves the groove separating the cusp edges. In this respect, therefore, the rheumatic commissural lesion of the aortic valve differs grossly from the generally rounded, smooth broad bridge of healing or healed subacute bacterial endocarditis, from the irregularly widened and eroded lesion of active subacute bacterial endocarditis, from the nodular and distorted fusion of the Mönckeberg type of aortic valve calcific sclerosis, from the separated margins of the syphilitic commissure, and from the depressed and almost obliterated raphe considered characteristic of the so-called congenital bicuspid aortic valve.

In spite of the striking picture presented by the rheumatic commissural lesion no reports are available on its pathogenesis. The present study concerns itself with this problem, together with the question as to whether or not, as in other cardiac sites (Gross *et al.*,[1-9]), the different clinical courses that rheumatic fever may take are reflected in the reaction of the commissural region to the inflammatory process.

MATERIAL AND METHODS

This report is based on a study of 70 rheumatic hearts, together with 50 normal hearts. As in previous reports, the rheumatic material was segregated into six groups representing the various clinical courses that the rheumatic process may run as follows:

GROUP I. Active cases where death took place during the first attack (9 cases).

GROUP II. Active cases where one preceding attack occurred within 1 year of the fatal outcome (9 cases).

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GROUP III. Active cases where one previous attack occurred at least 2 years previous to the fatal outcome (7 cases).

GROUP IV. Active cases with a history of repeated attacks, death occurring during an acute recurrence (11 cases).

GROUP V. Active cases where death was caused by decompensation without clinical evidence of a final recurrence. In some of these cases there was no previous history of rheumatic fever (25 cases).

GROUP VI. Inactive cases of chronic valvular disease of the typical rheumatic variety (9 cases).

The specimens were fixed in 10 per cent neutralized formol-saline, * standardized sections were taken according to the method of Gross, Antopol and Sacks,[10] and histological preparations were made using the technique employed by Gross and Ehrlich.[1] In a number of instances serial sections were made. In order to determine the optimum site and orientation of the sections for the study of these lesions vertical and horizontal serial sections were made through each of the aortic commissures in a number of rheumatic hearts. It appeared from these preliminary studies that microscopic changes were generally present in all the commissural regions and that these were more readily discernible in longitudinal cross sections (parallel with the long axis of the aorta). This section has the advantage of disclosing the aortic root, the aortic wedge (*i.e.* the wedge shaped termination of the aorta proper), the annulus extension of the aorta, the pericardial mantle and, occasionally, the aortic valve ring. For simplicity of approach the right-posterior commissure † of the aortic valve was chosen for study. This report will confine itself exclusively to this region.

* Solution of formaldehyde U. S. P. 10 parts, 1 per cent sodium chloride solution 90 parts. This solution is rendered neutral with a weak alkali.

† For terminology of these commissures and other cardiac sites see Gross, Antopol and Sacks,[10] and Gross and Kugel.[11]

Microscopic Appearance of the Normal Right-Posterior Commissure

(50 Cases)

A description of this region must take into account the age period changes in the topographical and histological arrangement of the aortic root, the aortic wedge (the apical portion of the root that becomes continuous with the aortic annulus), the aortic annulus, the aortic ring and subvalvular angle, and the pericardial mantle.

The aortic root, by which is meant the terminal 2 cm. of the aorta proper just prior to its termination as a wedge shaped structure (on cross section), displays capillaries in its outer third from approximately the first year on. These are generally circular on cross section, very inconspicuous, and in normal roots are never surrounded by inflammatory cells. From the middle of the first decade on, there appears to be a moderate increase in these rather inconspicuous capillaries. From the fourth decade on, the capillaries occasionally appear as dilated sinusoids, and rare lymphocytes may be present in their vicinity. During the later decades (sixth decade on) the capillaries are not infrequently even more numerous. This may be due to sclerotic changes which are frequently present during these age periods.

The aortic wedge (Fig. 1) displays capillaries from about the middle of the first decade. These are more apt to be of the wide sinusoid variety. Muscular wall vessels are infrequent. From about the fourth decade on, these sinusoids often appear to be more conspicuous at the boundary between the aortic wedge and the annulus extension. The musculo-elastic structure of the aortic wedge is generally quite regular until about the fifth decade when a variable amount of irregularity appears, due to the occasional penetration of irregular capillaries from the adventitial layer. This moderate distortion of the aortic wedge architecture should not be confused with scarring.

The annulus is normally devoid of capillaries and blood vessels. No inflammatory cells are present. The only age period change worthy of note is the development of hyalinization and the deposition of lipoid material in the later decades.

As has been previously described,[11] the aortic valve ring rarely contains capillaries, inflammatory cells are not normally present,

and the ring spongiosa is generally sharply defined until the later decades of life, when a certain amount of scarring takes place. No reduplications are present in the subaortic angle, although elastification in this site occurs in the later decades.

The normal structure of the pericardium has already been described by Friedberg and Gross.[7] Worthy of note in connection with the present study is the fact that the pericardial collagen fibers adjoining the adventitial layer of the aortic root only infrequently show a moderate amount of condensation. The pericardial layer external to this zone (Fig. 1) contains delicate fat septa, a moderate number of capillaries and nerve trunks, and an intact lamina propria. Scattered lymphocytes are occasionally noted throughout this adipose layer.

Microscopic Appearance of the Rheumatic Right-Posterior Commissure in Group I

(9 *Active Cases Where Death Took Place During the First Attack*)

In the description of the lesions present in this group and those subsequently to be discussed capillaries or cells will be considered as increased only when their incidence is greater than what one would expect to find normally in the corresponding age period.

Taken as a whole there was a distinct increase of capillaries in the aortic root in this group. These capillaries, surrounded by sparse lymphocytes, sometimes extended into the inner third of the aorta. Occasionally the aortic intima showed an inflamed reduplication (Gross [4]). The aortic wedge generally presented a marked increase in capillaries. These were almost invariably surrounded by inflammatory cells, chiefly lymphocytes, but occasionally also plasma cells and polymorphonuclear leukocytes. Incidentally, in referring to the increase in cells in this and other structures in all the groups under discussion it is to be noted that the lymphocyte is the most prominent cell and that plasma cells and polymorphonuclear leukocytes are generally present in considerably smaller numbers. The aortic wedge was moderately scarred (Fig. 2). This, however, was generally rather inconspicuous although it was occasionally quite severe. The aortic annulus extension as well as the ring annulus was frequently permeated with numerous capillaries surrounded by cells. These capillaries extended into the aortic ring and some-

times penetrated for a considerable distance down the annulus. The cells and capillaries in the aortic root, wedge, annulus and valve ring could be definitely traced as a contiguity process from lesions to be described in the pericardial mantle.

The rheumatic aortic valve ring lesions have been described in detail in a previous report.[8] Briefly, the most conspicuous changes found in this structure in the Group I cases were marked capillarization and inflammatory cell infiltration, slight scarring and inflamed subaortic angle reduplications.

Of considerable interest is the fact that in this group the pericardium almost invariably presented a dense collagenous zone of various thickness which merged imperceptibly with the aortic adventitia (Fig. 2). In about two-thirds of the cases there was present a moderate scattering of lymphocytes in this condensed zone. Less frequently, scattered lymphocytes were seen in the outer layers of the pericardium where, in addition, occasional thickenings of fat cell septa and capillary congestion were present. Almost invariably there could be observed lesions in the pericardial blood vessels. These vascular lesions were generally more conspicuous in the condensed collagenous zone referred to above and consisted of intimal fibrosis, intimal elastification, moderate hypertrophy and, on rare occasions, of intimal musculo-elastic hyperplastic changes.[3]

Summarizing these findings it appears that in Group I the commissural lesions consist of an increased capillarization of the aortic root, occasional inflamed reduplications of the aortic intima, somewhat more extensive capillarization and cellular infiltration of the aortic wedge with moderate scarring, permeation of the annulus extension and ring annulus by numerous capillaries and cells, active ring lesions, a zone of collagenous condensation in the pericardial mantle containing vascular lesions and lymphocytes, and moderate inflammatory changes in the other pericardial layers.

Microscopic Appearance of the Rheumatic Right-Posterior Commissure in Group II

(9 Active Cases Where One Preceding Attack Occurred Within 1 Year of the Fatal Outcome)

As in the valve rings and leaflets, the aortic commissure in this group presented considerably more dramatic changes than in

Group I. Capillarization of the root and wedge was much more extensive, inflammatory cells (chiefly lymphocytes) were quite conspicuous and the annulus showed a considerable increase in distorted capillaries, sometimes in muscular wall vessels. As in the previous group, the vessels and inflammatory cells showed a contiguity process that extended from the pericardial mantle, through the aortic root wedge and annulus into the aortic ring. Aortic intimal reduplications were frequently present.

While scarring of the aortic wedge was not as pronounced as in the groups to be subsequently described, it was quite definite in the majority of cases. Occasionally an isolated island or islands of musculo-elastic wedge tissue were cut off from the remainder of this structure.

The most conspicuous changes found in the aortic valve ring in the Group II cases were marked inflammatory cell infiltration and vascularization, the latter consisting frequently of vessels showing intimal musculo-elastic hyperplastic changes, pronounced scarring and multiple, inflamed, vascularized subaortic angle reduplications, frequently with verrucous change.

The alterations in the pericardial mantle were quite characteristic. The zone of collagenous condensation was very wide and considerably inflamed. The outer layers of the pericardium frequently showed the following changes: thickening of the fat septa, frequently with increased fibrosis, conspicuous infiltration with lymphocytes and striking vascular changes occurring throughout the entire pericardial mantle, consisting chiefly of medial hypertrophy, intimal fibrosis and elastification, giant medial hypertrophy, intimal musculo-elastic hyperplastic changes and granular plugging of the vessels. Occasionally the surface of the pericardium presented a fresh fibrinous deposit.

Summarizing these findings it appears that in Group II the commissural lesions consist of marked capillarization and inflammatory cell infiltration of the aortic root, wedge, annulus, ring and pericardial mantle; a contiguity process can be traced from the pericardium to the aortic valve ring; muscular wall vessels occasionally penetrate the aortic root; the aortic wedge shows considerable scarring with isolation of musculo-elastic islands; intimal musculo-elastic hyperplastic vessels are found in the various lesions; the ring lesions are active and intimal musculo-elastic hyperplastic vascular

lesions and multiple subaortic reduplications are conspicuous; and the pericardial lesions are particularly active, consisting of a wide inflamed zone of collagenous condensation, widespread vascular lesions of various types and diffuse inflammatory cell infiltration.

Microscopic Appearance of the Rheumatic Right-Posterior Commissure in Group III

(7 Active Cases Where One Previous Attack Occurred at Least 2 Years Previous to the Fatal Outcome)

In this group the aortic root was also generally markedly capillarized and sometimes vascularized with muscular wall vessels. Cellular infiltrations were, however, usually quite mild. The aortic intima presented multiple, generally elastified reduplications in approximately half of the cases.

The aortic wedge was frequently considerably scarred with resulting severe distortion of the wedge architecture and, sometimes, nipping off of musculo-elastic islands (Fig. 3). The deformity of the wedge was occasionally so severe that a semblance of elastica whorling was produced. The bands of scar tissue often contained blood vessels which extended from the pericardium. These were frequently of the intimal musculo-elastic hyperplastic type. As in the first group, the wedge lesions were much more severe than those in the aortic root and the cellular infiltration was usually mild.

The annulus lesion consisted of a conspicuous permeation with capillaries and, sometimes, with muscular wall vessels. These lesions were associated with considerably more cellular exudate than that found in the aortic root or wedge. The annulus lesion was generally extensive and often assumed an appearance suggestive of collagen whorling (Fig. 3).

The ring lesions which were also not infrequently in distinct contiguity with the annulus and, through this structure, with the pericardium, consisted briefly of the following: an almost invariable but at times somewhat milder exudative lesion with capillarization and vascularization with muscular wall vessels, and the invariable presence of inflamed, generally multiple, vascularized subaortic reduplications.

In contrast to the previous group, cells were rather infrequent in the pericardium. The zone of collagenous condensation referred to

above was much less conspicuous and was frequently absent (Fig. 3). In about half the cases the fat septa showed moderate thickening. Blood vessel changes were frequently found in the pericardium and consisted generally of medial hypertrophy, intimal fibrosis and sometimes of intimal musculo-elastic hyperplastic changes.

Summarizing these findings it appears that in Group III the commissural lesions consist of marked capillarization and sometimes vascularization (with muscular wall vessels) of the aortic root, wedge and annulus, mild cellular exudate in these structures, pronounced scarring of the aortic wedge with isolation of musculo-elastic islands, distortion of wedge elastica and annulus collagen producing lesions that resemble whorling, contiguity extension of the vascular lesions from pericardium to ring, multiple elastified reduplications of the aortic intima, moderate activity of the ring with multiple, vascular inflamed reduplications, and somewhat milder pericardial lesions with either inconspicuous or no collagenous condensation zone formation.

Microscopic Appearance of the Rheumatic Right-Posterior Commissure in Group IV

(11 Active Cases With a History of Repeated Attacks, Death Occurring During an Acute Recurrence)

In this group the aortic root also almost invariably presented increased capillaries which sometimes penetrated into the inner third of the aorta. Scatterings of lymphocytes around these vessels were rare. The aortic wedge was frequently richly vascularized. This vascularization differed from the previous three groups in that it generally consisted of thick muscular walled vessels. These also extended from the pericardium. Although inflammatory cells were infrequent in the aortic wedge they were somewhat more numerous than in the aortic root. Scarring of the aortic wedge was variable and was often quite severe. The intima of the aorta almost invariably showed multiple reduplications (Fig. 4). The annulus extension was usually permeated with distorted capillaries. At times these were infrequent. Cellularity was mild. The annulus lesion frequently extended for a considerable distance in all directions.

In brief, the ring lesions consisted of considerable vascularization, sometimes with intimal musculo-elastic hyperplastic changes but with very few inflammatory cells; at times only a few capillaries were found in the ring, and the subaortic angle almost invariably presented multiple elastified reduplications.

The zone of collagenous condensation in the pericardium was generally very broad and occasionally involved the entire thickness of the pericardial mantle (Fig. 4). Inflammatory cells were infrequent and when present were generally confined to the condensed pericardial zone. The fat septa were often thickened or congested. The pericardium almost invariably showed vascular changes consisting of medial hypertrophy, intimal fibrosis and occasionally giant medial hypertrophy or intimal musculo-elastic hyperplastic changes. These vessel changes were generally confined to the zone of collagenous condensation.

Summarizing these findings it appears that in Group IV the commissural lesions consist of increased capillarization of the aortic root, conspicuous vascularization of the aortic wedge with thick muscular walled vessels and a variable amount of scarring, permeation of the annulus with distorted capillaries, mild inflammatory cell infiltration of these lesions, multiple elastified reduplications of the aortic intima and subaortic angle, mild but vascularized ring lesions, and broad collagenous condensation zones in the pericardium, with mild cellularity but fairly advanced vascular changes.

Microscopic Appearance of the Rheumatic Right-Posterior Commissure in Group V

(25 Active Cases Where Death Was Caused by Decompensation Without Clinical Evidence of a Final Recurrence. In Some of these Cases There Was No Previous History of Rheumatic Fever)

Group V represented a considerably milder clinical course of rheumatic fever and, as with lesions previously described in other cardiac sites, was associated with much less alteration in the aortic commissures. Thus, the aortic root generally showed fewer capillaries than in the preceding groups, although occasionally capillarization was quite extensive. Lymphocytes, while generally present, were extremely inconspicuous. In approximately one-third of the

cases the aortic intima presented flat elastified reduplications. Capillarization of the aortic wedge was generally greater than that of the root. These capillaries were often associated with a variable amount of scarring. In about one-fourth of the cases the scarring was severe and resulted in elastica whorling (Fig. 5); in approximately one-fourth of the cases no scarring was present. No increase in inflammatory cells was noted in the aortic wedge in about half of the cases. When present they were sparse and somewhat more conspicuous than in the aortic root.

The annulus was generally much freer from inflammatory changes than in previous groups. In approximately two-thirds of the cases the annulus presented capillaries, occasionally muscular walled vessels. In a few cases the vessels were confined to the annulus-wedge boundary. Cells were infrequent. Although generally mild, the annulus lesion was sometimes quite widespread and associated with collagen whorling.

The ring lesions were considerably subdued in this group and consisted generally of thick walled muscular vessels or distorted capillaries, advanced scarring, sparse cellularity, and multiple elastified vascularized reduplications in about half the cases.

In this group the pericardium differed from that seen in the other groups in that there was found no zone of collagenous condensation in the majority of cases (Fig. 5). When present it was generally narrow. Lesions of the pericardial vessels were found less frequently than in the previous groups. When present they occurred chiefly in the narrow zone of collagenous condensation and consisted of medial hypertrophy, intimal elastification and occasionally giant medial hypertrophy. In about half of the cases no vascular lesions were seen. Cellularity was most inconspicuous and when present the lymphocytes were scattered between the fat cells rather than in the collagenous zone of condensation.

Summarizing these findings it appears that in Group V the aortic root and wedge show fewer capillaries than in the preceding groups; flat aortic intimal reduplications occur only in one-third of the cases; scarring of the aortic wedge is variable but generally less conspicuous than previously described; inflammatory cell infiltration is very mild; the annulus lesions are similarly less involved but there is a tendency for vessels to be more prominent at the annulus wedge boundary; ring lesions contain thick walled vessels and distorted

capillaries, but few inflammatory cells; the pericardium rarely shows a collagenous condensation zone; and vascular lesions and cell infiltrations are rather inconspicuous.

Microscopic Appearance of the Rheumatic Right-Posterior Commissure in Group VI

(9 Inactive Cases of Chronic Valvular Disease of the Typical Rheumatic Variety)

The only consistent alteration in this group was a moderate increase in capillaries in the aortic root with mild scatterings of lymphocytes. No reduplications were noted in the intima. The aortic wedge showed slight scarring in about half the cases and capillaries were somewhat more numerous at this site. In 1 case the wedge was penetrated by muscular walled vessels. When capillaries were present in the wedge they were generally distorted. Inflammatory cells were infrequent. The annulus showed capillarization in only 2 cases but no inflammatory cells were present. When lime was present near the aortic ring there occurred a considerable increase in blood vessels, capillaries and cells. Otherwise the valve rings were generally severely scarred and almost completely devoid of inflammatory cells. When vascularization occurred it consisted of capillaries, hyaline arterioles and hypertrophied vessels. In half the cases multiple elastified subaortic reduplications were present.

One of the most characteristic features of this group was the extreme paucity of pericardial lesions behind the commissure. Thus, zones of collagenous condensation were infrequent and, when present, were narrow and irregular; lymphocytes were noted in only 2 cases and in these they tended to be concentrated in the outer layers of the pericardium; and vascular lesions were not observed. In most cases the septa between the fat cells were somewhat thickened.

Summarizing these findings it appears that in Group VI the aortic root presents a moderate increase in capillaries but no intimal reduplications; the aortic wedge shows slight scarring in about half the cases, and a somewhat greater increase in capillaries; the annulus is relatively free of inflammatory lesions; inflammatory cells are infrequent in these sites; the ring lesions are practically completely inactive, about half the cases showing only vestiges of previous in-

flammation in the form of severe scarring, capillarization, thickened arterioles and muscular vessels, and subaortic multiple elastified reduplications; and the pericardial mantle is relatively free of inflammatory alterations.

Discussion

A brief review of the inflammatory changes found in the aortic commissures of the six groups herein described reveals a remarkable parallelism with the clinical course of the rheumatic disease, and thus agrees closely with the findings previously described in other cardiac sites. Commissural lesions were most conspicuous and active in the first four groups, diminished strikingly in activity in the fifth group, and existed only as vestiges in the sixth.

The first group showed considerable capillarization of the aortic root and its extensions, fairly marked inflammatory cell infiltration, relatively little scarring, and inflammatory cells in the pericardium with a zone of collagenous condensation and vascular changes. The second group differed in the somewhat more extensive inflammatory changes, the appearance of muscular walled and intimal musculo-elastic hyperplastic vessels in various sites, greater scarring of the aortic wedge with nipping off of musculo-elastic islands, extraordinarily active ring lesions, and intensely inflamed pericardium with a wide collagenous condensation zone, and vascular lesions in great variety. The third group was somewhat similar to the second except that the aortic intima presented multiple reduplications, inflammatory cell infiltration was less severe, the pericardial collagenous condensation zone was usually narrower or absent, and the pericardial vascular lesions not so varied. The fourth group also showed a mild grade of exudative phenomena, the aortic wedge was frequently vascularized with muscular wall vessels and the pericardial collagenous condensation zone was very broad. In Group V inflammatory cells were quite sparse, the aortic root intimal reduplications were single, flat and less frequently present, wedge lesions were less severe, the annulus lesions were less frequent and the pericardial lesions were quite mild. In Group VI activity was practically nil; when present the lesions were chiefly confined to the aortic wedge and the pericardium was relatively free of alterations but inconspicuous vestiges of inflammatory lesions could sometimes be found in the root, wedge and ring.

The topographical features of some of these lesions, together with the constancy of the findings, clearly indicate the pathogenesis of the commissural lesion. In a previous report Gross and Friedberg [8] described several avenues through which the valve rings may be affected. It was shown that one of these is by way of the adjacent pericardial mantle which is in intimate relation to the valve rings in the region of the great vessel roots.[11] In a recent report by Gross [4] it was shown that the great vessel roots are frequently the seat of inflammatory disturbances during the course of a rheumatic process. It was indicated in this report that the pericardium may play a not insignificant rôle in the initiation and continuation of inflammatory phenomena at this site. These observations receive further confirmation through the findings described in this report. As mentioned above, in all the active rheumatic groups the pericardial mantle of the aortic valve commissure showed pronounced inflammatory changes. These consisted of zones of collagenous condensation representing organization of inflammatory tissue, inflammatory cells varying in amount with the acuity of the process, conspicuous changes in the blood vessels of the pericardium, and penetration of these vessels into the aortic root, aortic wedge and annulus. From the latter site the penetration could be traced directly to the aortic ring where the inflammatory process generally appeared to be most active. Furthermore, there was present a large graded series of lesions which presented all stages in the penetration of granulation tissue from the pericardium inward toward the ring. Thus, although it cannot be denied that the initial rheumatic infection may reach the valve ring from other sources, such, for example, as the intervalvular fibrosa or the underlying myocardium, and spread in retrograde fashion to the adjacent pericardium, the mechanism of granulation tissue penetration into the rheumatic aortic ring is undoubtedly chiefly by contiguity from the latter site. Granted, then, infection in the aortic valve ring, with coincidental or subsequent granulation tissue permeation, this spreads distally into the valve leaflets and, because of their close apposition at the commissural region, leads to agglutination of their edematous and inflamed ventricularis mantles. Apparently the constant apposition of these leaflets and, possibly, the additional trauma imposed on them by their systolic and diastolic movements, lead to increased inflammatory phenomena with agglutination and organization.

It is of considerable interest that the clinical course of the disease is accurately depicted by the qualitative and quantitative differences in the commissural lesions described above. The presence of these lesions in very high incidence constitutes another group of stigmata by which extinct rheumatic fever may be recognized, for it has been shown that even in the completely indolent Group VI evidence of such past infection can sometimes be observed.

The lesions of the aortic root have already been described in considerable detail elsewhere.[4] In the present studies it is shown that scarring of the aortic wedge with distortion of the normal architecture is of frequent occurrence in rheumatic fever and that in the more active groups the distortion may become quite severe. That it never reaches the extreme grade of destruction seen in syphilis (Fig. 6) differentiates the rheumatic lesion very easily from the former and also affords an explanation for the absence of the characteristic commissural separation seen so frequently in aortic root syphilis. On the other hand, it is not inconceivable that a marked grade of destruction in the aortic wedge produced early in life may so completely distort the normal topography of this structure that there may be produced a picture suggestive of faulty inversion such as that described by Lewis and Grant [12] in the congenital bicuspid aortic valve. In this connection it is of considerable interest that elastica whorling of the wedge apex and collagen whorling of the annulus were not infrequently encountered in the severe lesions. These have also been considered indicative of a congenital lesion by the above mentioned authors. In the light of these findings, therefore, it seemed advisable again to study the pathogenesis of the so-called congenital bicuspid aortic valve from the point of view of the possible rôle played by inflammation, chiefly of the rheumatic variety, in the production of this lesion. This matter forms the basis of a separate report which has been published elsewhere.[13]

Summary and Conclusions

There has been described in this report the findings in 70 cases of rheumatic fever segregated into six groups according to the clinical course taken by the disease. It is shown that a number of inflammatory changes are found in the aortic root, wedge, annulus, ring, subaortic angle and pericardial mantle which are characteristic of rheu-

matic fever and, to some extent, reflect the clinical course of the disease. Even when healing takes place the histological characteristics of the commissural lesion afford additional stigmata which are of value in discerning a past rheumatic process. A discussion is given of the pathogenesis of this lesion from which it appears that even though the original infection may reach the aortic ring through several routes, in most instances the inflammatory granulation tissue passes from the pericardial mantle through the aortic root, wedge and annulus to reach the aortic rings. The latter show a much more flagrant inflammatory process which spreads into the valve leaflets and, probably with the additional factor of trauma caused by the systolic and diastolic movements of the cusps, eventually leads to their agglutination. The possible significance of these findings in relation to the pathogenesis of the so-called congenital bicuspid aortic valve is indicated. A description is also given of the histological and topographical changes taking place during the different age periods in the normal aortic commissural region.

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DESCRIPTION OF PLATES

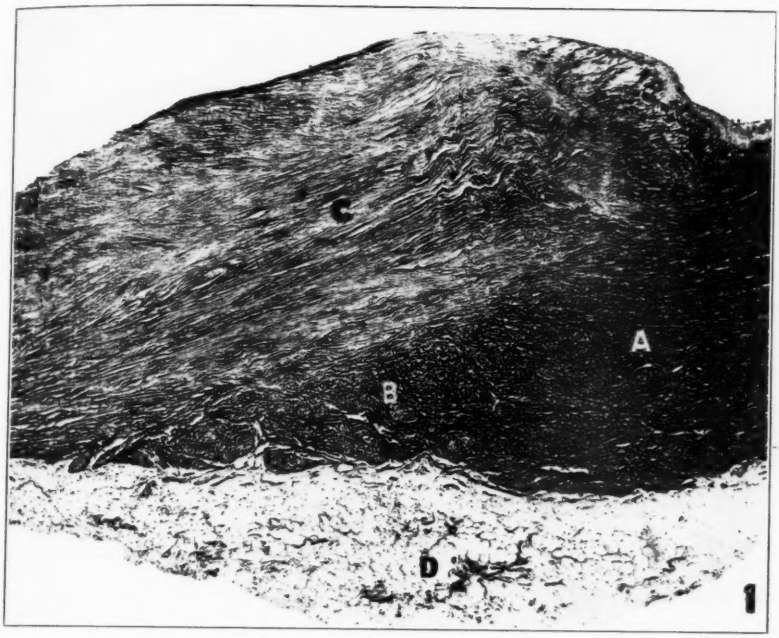
PLATE 60

FIG. 1. Longitudinal cross section through the right-posterior aortic valve commissure from a normal control case. Age 50 years. Low power. Weigert's elastic and Van Gieson's connective tissue stain.

A = aortic wedge; B = sinusoidal capillary; C = ring annulus; D = pericardium.

FIG. 2. Longitudinal cross section through the right-posterior aortic valve commissure from a rheumatic case classified as Group I. Age 9 years. Low power. Weigert's elastic and Van Gieson's connective tissue stain.

A = aortic wedge; B = scarred apex of aortic wedge with penetrating capillaries; C = ring annulus containing few capillaries; D = capillarized annulus extension. Note penetration of capillaries from pericardium. E = capillarized aortic ring; F = subaortic reduplication; G = capillarized inflamed reduplication in aortic sinus pocket; H = collagenous condensation zone in pericardium; J = blood vessel showing intimal fibrosis; K = inflamed adipose tissue.



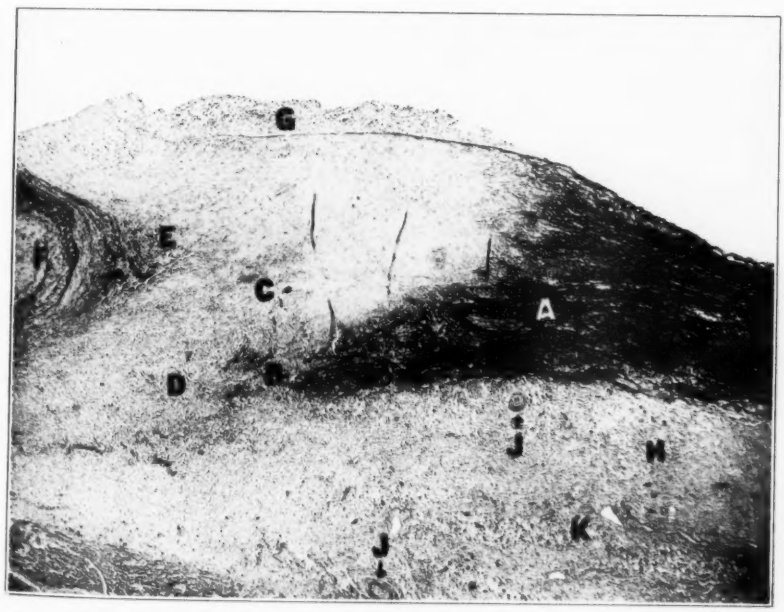


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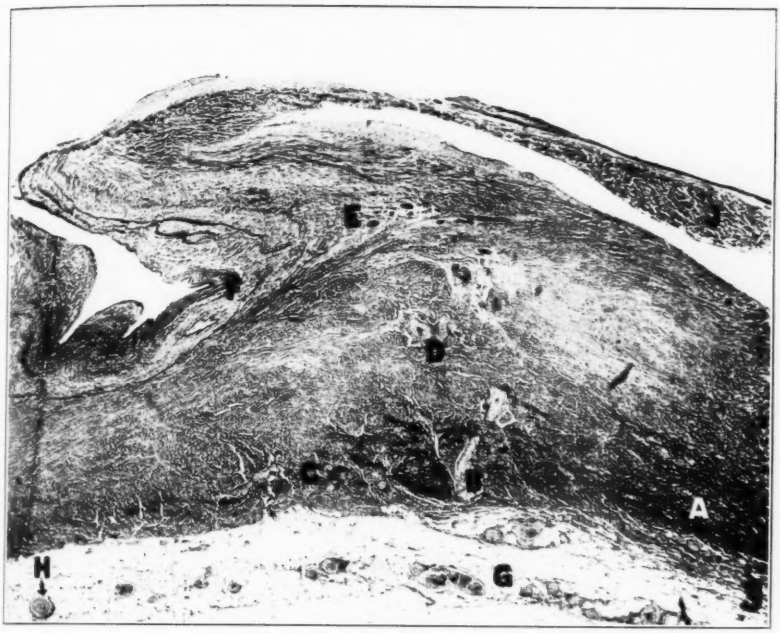
PLATE 61

FIG. 3. Longitudinal cross section through the right-posterior aortic valve commissure from a rheumatic case classified as Group III. Age 19 years. Low power. Weigert's elastic and Van Gieson's connective tissue stain.

A = aortic wedge; B = penetrating muscular wall vessel with scar tissue nipping off musculo-elastic island; C = almost complete obliteration of wedge apex. Note numerous blood vessels. D = vascularized ring annulus; E = vascularized ring; F = vascularized subaortic reduplications; G = pericardium containing altered blood vessels. Note absence of collagenous condensation zone. H = blood vessel showing intimal musculo-elastic hyperplastic changes; J = posterior aortic leaflet.

FIG. 4. Longitudinal cross section through the right-posterior aortic valve commissure from a rheumatic case classified as Group IV. Age 20 years. Low power. Weigert's elastic and Van Gieson's connective tissue stain.

A = aortic wedge; B = capillarization and scarring of aortic root; C = multiple aortic intimal reduplications; D = capillarized ring annulus; E = collagenous condensation zone in pericardium; F = altered blood vessels.



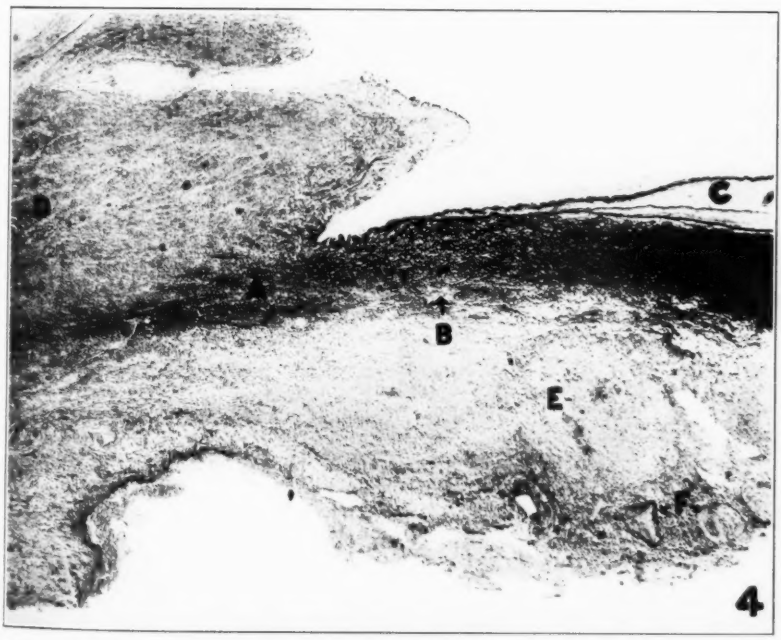


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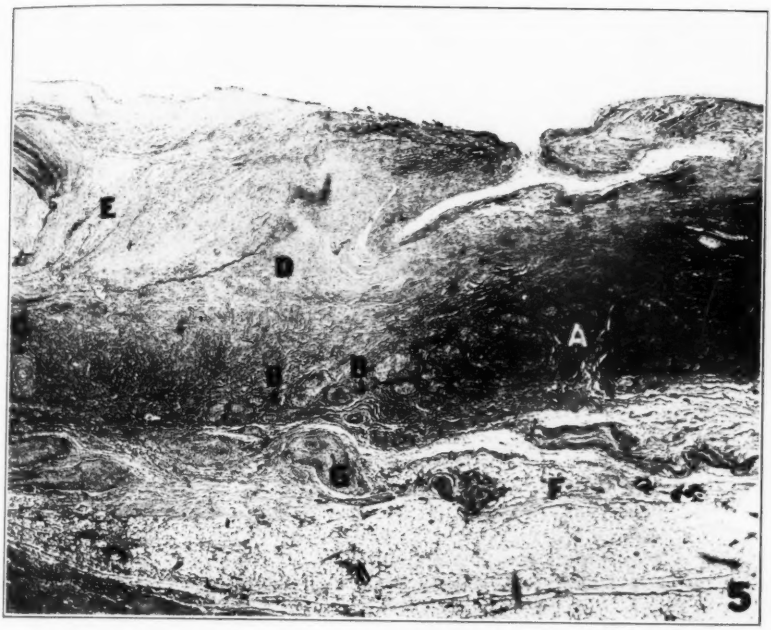
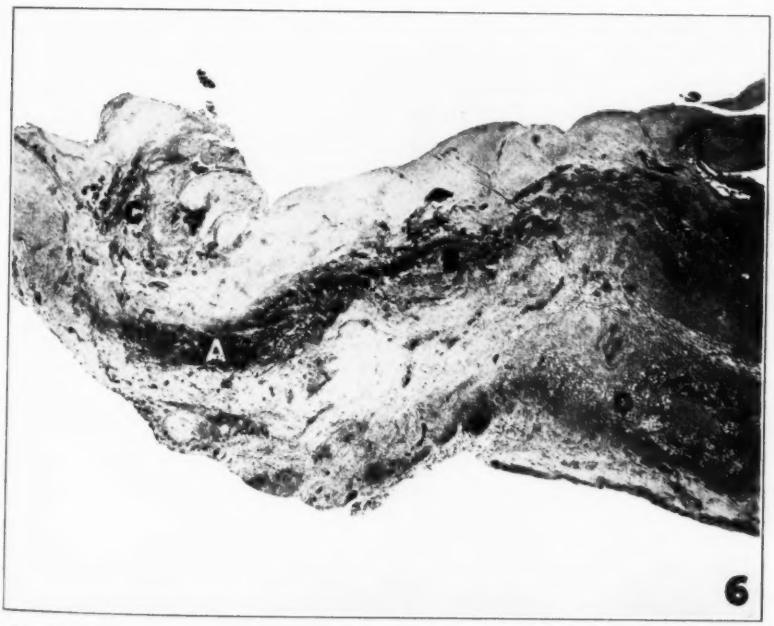
FIG. 5. Longitudinal cross section through the right-posterior aortic valve commissure from a rheumatic case classified as Group V. Age 20 years. Low power. Weigert's elastic and Van Gieson's connective tissue stain.

A = severely distorted aortic wedge due to penetrating blood vessels and scar tissue; B = scar tissue nipping off musculo-elastic islands; C = muscular walled vessels in annulus extension; D = scarred ring; E = vascularized subaortic reduplications; F = pericardium containing (G) enormously hypertrophied vessels with intimal fibrosis. Note absence of collagenous condensation zone.

FIG. 6. Longitudinal cross section through the right-posterior aortic valve commissure from a case with aortic root syphilis. Age 37 years. Low power. Weigert's elastic and Van Gieson's connective tissue stain.

A = distorted aortic wedge; B = severe scarring of aortic wedge and root producing almost complete destruction of elastic and muscular tissue; C = severe ring lesion; D = pericardium showing intense inflammation with severe vascular changes.





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THE MINERAL CONTENT OF VARIOUS CEREBRAL LESIONS AS DEMONSTRATED BY THE MICROINCINERATION METHOD *

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INTRODUCTION

Microincineration was first applied to botanical studies by Raspail [1] in 1833. Liesegang [2] in 1910 studied by means of this method the mineral residue of blood in the capillaries of embryonic brain, lens, retina and optic nerve of fish. Herrera [3] (1913), Naumann [4] (1915), Prenant [5] (1919), and Molisch [6, 7] (1920–1921) made use of it in various botanical and zoological problems. In 1923 Policard [8] devised newer and more detailed methods of procedure and observation, and together with co-workers introduced the method into human histopathology.[9–13] Later Scott [14–16] revised and improved the method, especially by adding darkfield to Policard's observations in oblique light.

With this method the normal anatomy and pathology of many body tissues of man, vertebrates and invertebrates were studied and many fundamentally new observations were made. Clear recognition of silica (Herrera,[17] Lanzoni [18]) enabled Policard and Doubrow,[19] and later Lecloux,[20] to demonstrate associated silicosis in anthracosis of the lungs and thereby to interpret it in a new light. Policard and Pillet [21] discovered calcium in the liquid contained in the intracranial diverticulums of the *saccus endolymphaticus* in frogs, and Baginski [22] demonstrated the calcium content of cartilage, also hyaline cartilage, which had not been demonstrable by staining methods. Kooyman [23] studied the minerals in the skin. Policard, Noël and Pillet,[24] Okkels,[25, 26] and others, studied changes in the

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mineral structure of organs produced by metabolic changes: after a carbohydrate diet mineral ash increases in the peripheral parts of liver lobules; in experimental calcium poisoning this mineral is deposited in the convoluted tubules and papillae of the kidney; and injected iron salts are deposited in the liver, spleen and kidneys in inverse order of their solubility.

The important question whether minerals in tissues, as demonstrated by microincineration, actually correspond in their arrangement to vital structures or not was successfully answered by Scott,[27] who proved their identity with the materials revealed by ultraviolet light photomicrography of living cells (Köhler,[28] Lucas,[29] Wyckoff and Ter Louw,[30] Lucas and Stark,[31] and Wyckoff, Ebeling and Ter Louw[32]). Scott also refers to the fact that for nuclei this had already been demonstrated by Policard.[33] This has also been confirmed by the studies of Gage, Day and Starrett.[34]

More difficult is the question of identification of the minerals observed. With certainty only three mineral elements can be identified, namely iron, calcium and silica. Policard,[35] in one of his first papers on the subject, found that by microincineration all iron compounds, including organic compounds — the so-called "masked iron" — are transformed into iron oxide ($Fe_2 O_3$), which stands out by its light yellow to dark red color. Where only a slight amount is present it is light yellow, as in fluid blood in vessels; where it is abundant the color is vivid red, as in phagocytic cells or in old hemorrhages. Scott [15] and others confirmed Policard's finding. Scott points to an important source of error in interpretation: in darkfield illumination carbon particles remaining in incompletely incinerated preparations also give a yellowish to red color; however, these carbon particles when viewed with transmitted light will appear black, while only iron oxide retains its color. Therefore it is necessary, before interpreting any colored particles to be iron, to ascertain by transmitted light whether or not the preparation has been completely incinerated. Microincineration, applied with this caution, is superior in accuracy of demonstrating iron in tissues to any known staining reaction (Scheid,[36] Marza, Marza and Chiosa [37]), even to Macallum's [38-40] methods.

Silica is present as a white, doubly refractive, crystalline mineral insoluble in water. Scott,[15] however, points to the fact that in all probability it is present as a silicocalcareous compound, and

therefore quantitatively its actual amount can easily be overestimated.

Calcium is present as a bright, white, monorefractive ash, which is practically insoluble in water (Schultz-Brauns [41]), although a small quantity of it may go into solution (Sheldon and Ramage,[42] Scott [16]). It may, furthermore, be ascertained by the gypsum or the oxalic acid reaction (Ostertag [43]). For interpretation of the distribution of calcium salts in tissues microincineration was most revolutionary and revealing. In many tissues (*i.e.* blood vessels, placenta) the usual staining reactions for calcium (Röhl-von Kóssa-hematoxylin) show only a relatively small part of the total amount of insoluble calcium salts, as demonstrated by microincineration (Schultz-Brauns and Schoenholz,[44] Schoenholz,[45] Zinkant [46]). In other instances, however, microincineration reveals the absence of mineral where the staining reactions seem to indicate the presence of calcium. According to Scott [15] the usual histochemical tests for calcium reveal it in large quantities in and about the parietal cells of gastric glands, yet microincineration, which offers but slight chance of any appreciable alteration in the location of minerals in cells and tissues, shows that little, if any, salt is present in these cells except in their nuclei.

The recognition of magnesium in microincinerated slides is unsatisfactory, especially in the presence of calcium (Scott [15]). Although attempts have been made to identify sodium and potassium (Jacobi and Keuscher,[47] Policard and Pillet [48]) their recognition is still unsatisfactory in spite of the fact that much less of these elements is volatilized by the heat of microincineration (Sheldon and Ramage,[42] Scott [16]) than previously assumed. According to Schultz-Brauns [41] the ashes of sodium, potassium and magnesium can be differentiated from the ash of calcium by their bluish hue, but although this may give practically useful clues it should be remembered that transmitted structural blue is due to smallness of quantity rather than to any inherent quality (Mason,[49] Scott [16]).

The microincineration method can be used only for estimation of the relative quantity of mineral ashes. It should be borne in mind that microincineration is a microscopic method, and still more specifically an ultramicroscopic method. Attempts to force quantitative chemical information (Schultz-Brauns,[50] Wulf,[51] Marza and Chiosa [52]) were doomed to failure. Microincineration at present

gives accurate information only as to the location of the total heat-resistant minerals and specifically of iron, silica and calcium in cells and tissues, lacking in other histochemical procedures; or, as Scott [15] concludes, "Observation of incinerated preparations gives an exact conception of the mineral structure of protoplasm." Finer analyses will have to be made by spectroscopic and chemical examination (Gerlach and Schweitzer,[53] Gerlach and Gerlach,[54, 55] Gerlach,[56] Policard,[57] Scott and Williams [58]).

Data on the nervous system, obtained by the microincineration method, are still scarce. Scott [15] studied the localization of mineral salts in the normal nervous system of mammalia. Of pathological lesions of the nervous system the best known as to mineral structure are inclusion bodies. Covell and Danks [59] found that Negri bodies in rabies contained a rather compact ash residue; intranuclear inclusions from the cerebral cortex of rabbits in herpes (Rector and Rector [60]), however, contain mineral only in their early stages and lose it later. In contrast to these the intranuclear inclusions in the submaxillary glands of guinea pigs (Cole and Kuttner,[61] Scott [62]), according to Scott,[63] and the intranuclear inclusions in yellow fever, according to Cowdry,[64] do not contain mineral. The inclusion bodies in fowl-pox were found to contain mineral by Danks.[65]

Scheid [66] described loss of mineral residue in granular cells of the cerebellum during diabetic coma. He also described the mineral structure of perivascular hemorrhages in the brain. Wulf [51] described iron in the vessel wall and in perivascular scavenger cells in a case of old perivascular hemorrhage. In an organized thrombus from a vessel of the human pons he described the net-like ash structure of the thrombus and iron in the wall of the thrombosed vessel. He emphasized that the pseudocalcium of vessel walls does not leave ash residue. He confirmed Scheid's finding of disappearance of the ash in the granular layer of the cerebellum in 2 cases of coma diabeticum, but points out, however, that in other of his cases (uremia, arteriosclerosis) the granular layer was poor in ash. Patton [67] studied the cell changes in experimental poliomyelitis and found that after a transitory stage of increase of minerals the diseased anterior horn cells became demineralized.

These findings seemed significant enough to warrant undertaking a systematic study of various cerebral lesions on a larger series of material.

Methods and Material

In our studies we followed Scott's [15, 16] modification of the method and the general technical rules laid down by him.*

The microincineration technique consists in exposing sections of tissue fixed in absolute alcohol and formalin,† embedded in paraffin,§ and cut at 4 μ, to temperatures gradually rising to 650° C. By this procedure the organic components of the section are burnt out and only the mineral ash, or heat resistant mineral constituents of the tissue, remain on the slide. At low power the preparations are viewed with oblique transillumination, at high power by dark-field illumination. By this method the tissue minerals that are not volatilized or vaporized at temperatures up to 650° C. are demonstrated. These are the *heat resistant mineral ashes*, which make up an important portion of the mineral constituents of mammalian and human tissues.

Neighboring sections of all incinerated sections were cut and stained with one of the usual stains (cresyl violet, hematoxylin-eosin, Masson's trichrome stain, and some with a modification of Bielschowsky's silver impregnation for paraffin sections).

* We are also obliged to Dr. Scott for having a furnace constructed in his laboratory and sent to us, which we used for most of our work.

In the latter part of our work we also used furnaces which are now manufactured by the A. S. Aloe Company in St. Louis, according to Dr. Scott's directions.

† From tissue fixed in 95 per cent alcohol satisfactory preparations can be obtained. Tissue fixed in 10 per cent formalin also gives comparable, though less brilliant pictures, if the blocks have not been left in formalin for more than 2 to 3 weeks. If possible, Scott's original fixative (absolute alcohol 9 parts, 40 per cent formalin 1 part) should always be chosen as it is superior to any other fixative in the brilliancy and completeness of the ash picture, and in avoiding any irregular shrinkage.

§ In a few cases additional frozen sections of 10 to 16 μ from the same material (fresh and fixed) were incinerated. This procedure, though the pictures obtained corresponded essentially to those obtained from paraffin embedded material, at least at low power, has two distinct disadvantages: (1) a greater tendency to incomplete incineration of the tissue; and (2) lack of cytological detail. Both these disadvantages are due probably to the necessarily greater thickness of frozen sections. The results, however, were somewhat more satisfactory when frozen sections, before incineration, were passed through graded alcohols and ether.

From thin celloidin sections, 4 to 8 μ, quite satisfactory pictures may be obtained. It is necessary, however, to dissolve the celloidin completely in alcohol and ether before incineration.

The most satisfactory method, however, is the use of paraffin sections cut at 4 μ. All the descriptions and conclusions in our study are based on such sections and with exceptions of Figure 34 the illustrations are taken from them.

Our material consisted of brains from the following 40 human cases: *

Normal adult (death from lobar pneumonia)	2
Newborn premature	2
Cerebral arteriosclerosis	9
Senile and presenile atrophy (dementia)	
Alzheimer's disease	4
Pick's disease	2
Syphilitic meningoencephalitis and dementia paralytica	4
Purulent meningitis (pneumococcus) with purulent meningoencephalitis	1
Chronic alcoholism (Korsakoff's psychosis and delirium tremens)	3
Multiple sclerosis	2
Diabetic coma	2
Symptomatic psychosis	
in cardiorenal disease	1
in pneumoconiosis	1
Traumatic laceration of brain	1
Manic depressive psychosis	
complicated by emaciation	1
complicated by pulmonary and intestinal tuberculosis	1
Schizophrenia	
complicated by emaciation	1
complicated by pulmonary and intestinal tuberculosis	1
Brain tumor	
Glioma (astroblastoma)	1
Metastases from carcinoma of bronchial epithelium with squamous metaplasia	1

From these cases the following cortical areas were examined: central region (areas 4, 6, 3, 2, 1), area frontalis granularis (10), area calcarina (17, including parts of 18), Ammon's horn, and area entorhinalis. In addition to these cortical areas we examined blocks from the striatum, thalamus and hypothalamus, cerebellar cortex and medulla oblongata. In some cases we examined also sections from the spinal cord.

In addition to this human material we have been collecting several series of experimental animal material with normal control tissue from the same animals, including brains and body tissues of dogs, cats, rabbits, guinea pigs and pigeons.

* Nine of these cases were obtained from the autopsy material of the Mallory Institute of Pathology and the Neuropathological Laboratory of the Neurological Unit of the Boston City Hospital and 2 cases from the Worcester State Hospital, through the courtesy of Drs. F. Parker, Jr., and T. J. Putnam, and Drs. J. M. Looney and W. Freeman.

Mineral Structure of the Cerebral Cortex of the Newborn

The cerebral cortex of the human newborn is rich in mineral ash. Sections show a great amount of mineral in the nuclei of ganglion cells and glial elements of all cortical layers (Fig. 1). The cytoplasm of these cells, however, contains none or very little mineral ash. A slight yellowish hue here and there in the nuclear mineral ash is indicative of the presence of iron; however it is far surpassed in relative amount by calcium and probably also, although in lesser degree, by some water soluble minerals. These findings show that minerals are inborn in the cerebral cortex of man. These minerals however are, in contrast to the arrangement in adults, accumulated in the nuclei of ganglion cells. Also, other actively growing tissues show nuclear mineral deposits, as can be seen from the literature and from our studies.

This finding bears upon an important point, namely the biological significance of tissue minerals. It has been a common belief that minerals in the tissue appear especially where necrosis has occurred. Even in the beginning of the work on microincineration some authors still clung to this concept. Schultz-Brauns [41] considered deposits of calcium as occurring either in tissues and cells which are necrotic, or in cells with decreased function and metabolism, in the sense of Freudenberg and György.[68] In another paper Schultz-Brauns and Schoenholz,[41] although stressing the presence of mineral, and especially of calcium, in living tissue, *i.e.* in living placental epithelium and in migrating histiocytes of the chorion, still consider it as indicative of cell damage. They consider even the migrating histiocytes that contain calcium to be damaged. This interpretation is made in spite of the findings of Wehefritz [69] and Schoenig,[70] who from clinical, chemical and physiological points of view interpreted the presence of the larger quantities of calcium in early growing placentas as indicating a physiological storage of calcium. They considered calcium in the necrotic areas of placental infarcts pathological or degenerative in nature. In a later paper Schultz-Brauns [71] claims that calcium deposits in cortical ganglion cells of the cerebrum are pathological.

Scott [63] changed this concept by clearly and irrefutably demonstrating that minerals, including calcium, are an inherent part of

the nucleus of the actively growing and dividing cell, and in their arrangement correspond to the nuclear chromatin, especially in cells in mitosis (Scott,[14, 72] Funaoka and Ogata [73]). Recently Scott [74] confirmed this finding in tissue treated by the Gersh-Bensley method. Policard [75] found mineral, most of it calcium and magnesium, in the nuclei, and less of it in the cytoplasm of macrophages from tissue cultures. Horning and Scott,[76] studying tissue cultures of mesodermal cells, found no visible gradation in the mineral content between the elements nearer the original implant and those that migrated the farthest into the liquid medium, thus forming the growing edge of the culture. However, while tissue cultures contain a fair amount of calcium oxide and sodium they are practically free of iron. Clasmatocytes and other ameboid elements are the most outstanding in tissue cultures, owing to the enormous concentration of mineral salts (calcium) within the main body of the cell and in their pseudopodia. Policard [77] found the nuclei of the seminal cells and the spermatozoa in man and in rats rich in minerals. In the spermatozoa he found calcium, as well as traces of iron, the latter, however, in the anterior part of the head only. Marza, Marza and Chiosa,[37] and later Baginski,[78] found minerals but no iron in the nucleus of the ovum (oocytes and de Graaf's follicles). Iron in the ovary was found in two forms: (1) inorganic in the interstitial cells; and (2) organic in the vitellus of the ovum during the period of rapid growth. Horning and Scott [79] demonstrated the inorganic structure of the growing organism by studying the distribution and changes in inorganic salts during embryonic development of the chick. Embryonic development is inorganically expressed by an increase in certain mineral salts and a decrease in others. During growth of the brain there is a gradual increase in calcium content, while iron salts decrease. The growing cells, and especially their nuclei, were found to be rich in mineral residue.

Minerals in Ganglion Cells of the Cerebral Cortex, Cerebellar Cortex and Spinal Cord of the Normal Adult with a Discussion of their Pathological Alterations

In incinerated preparations of human cerebral cortex the layers of cortical ganglion cells stand out at low power like the negative of a Nissl preparation (Fig. 3), however, with one difference. This

consists in the fact that in the cerebral cortex granular cells contain much less mineral than large and small pyramidal cells. Therefore, the granular layers stand out as less mineralized than the pyramidal. The glial ground net stands out as a fine network containing slight traces of mineral (Fig. 9). The nuclei of glial cells show mineral residue which is most compact in the nuclei of the oligodendroglia cells of normal brain.

The mineral in cortical ganglion cells of the pyramidal type in the adult is restricted to the cytoplasm and nucleolus, while the nucleus itself is essentially free from mineral residue, except for fine, faintly visible strands, especially along the nuclear membrane (Fig. 9). The mineral in the cytoplasm is globular and granular in arrangement, extending into the apical processes and also into the roots of the dendrites. It corresponds in its arrangement to Nissl bodies. The axone hillock, axis cylinder process and the axone itself are free from mineral, a fact established by Scott.[15] Also, the intracellular neurofibrils are free of mineral. Most of the ash is white, some of it bluish; most of the white ash is insoluble in water. Here and there are granules, but never entire Nissl bodies, in which a slight yellowish tinge indicates the presence of iron. These iron-containing granules are mixed in with the white and bluish granules, frequently in the same Nissl body. There are no Nissl bodies entirely made up of yellowish (iron-containing) granules. The iron-containing granules are less numerous in cortical ganglion cells than in motor ganglion cells of the spinal cord, but even there they are not frequent and are far outnumbered by iron-free granules.

The pyramidal cells of the fascia dentata of the hippocampus are outstandingly rich in all minerals (Fig. 4).

In normal cerebellar cortex the richest mineral deposits are found in the granular cells, while the Purkinje cells contain a comparatively sparse amount in their nucleolus, cytoplasm and dendrites (Figs. 5, 6, 7 and 8). The molecular layer contains but little mineral deposit (Fig. 5) located in the glial Bergmann processes, in sparse glial nuclei and in the nucleoli and faintly visible, short processes of scattered small ganglion cells of the molecular layer (Fig. 6). In suitable preparations long dendrites of the Purkinje cells are well outlined by their mineral content (Fig. 7); the axis cylinder, however, is always invisible, being free of mineral. The intracellular neurofibrils are free of mineral. In some instances Cajal's baskets

are well outlined by the mineral granules they contain (Fig. 8). A part of them is made up of myelinized fibers (Obersteiner [80]). Others are probably dentrites from basket cells, since axis cylinders themselves do not contain mineral ash.

The finding of richer mineral deposits in granular cells in the cerebellum is in contrast to that in the cerebrum where they contain the least amount of mineral. This indicates that granular cells in the cerebrum and cerebellum are fundamentally different in their chemical composition.

In 2 cases of diabetic coma (diabetes mellitus), which we examined, the cerebellum gave in all its parts a typically normal incineration picture. Therefore, we are unable to confirm the statements of Scheid [66] and Wulf [51] concerning a diminution or disappearance of mineral matter from the granular layer of the cerebellar cortex in this condition.

In the spinal cord large motor ganglion cells of the anterior horn stand out by the substantial mineral deposits in their cytoplasm and nucleolus (Figs. 2, 10, 11, 12, 13), while the nucleus is free of them, except for faintly visible fine strands found especially along the nuclear membrane. The mineral deposits in the cytoplasm are globular and, as demonstrated by stained control sections, correspond in their arrangement to Nissl bodies (Figs. 10, 11, 12). The dendrites are also rich in minerals (Fig. 10), while the axis cylinder and axone hillock have none (Fig. 13). Intracellular neurofibrils in normal cells are also free of minerals. That within the Nissl bodies is granular in nature. Most of these granules appear white in darkfield, some bluish. Most of the white granules are insoluble in water. In addition to these the Nissl bodies contain a few granules which by their yellowish tinge indicate the presence of iron. These yellowish granules are somewhat more numerous in motor ganglion cells of the spinal cord than in cerebral cortex. The nucleus is free of iron. The nucleolus is composed of dense, white, water insoluble ash, some of it doubly refractive, as already observed by Scott [15]; whether or not the nucleolus also contains iron cannot be decided with certainty.

Nicholson [81] in 1923, working with Macallum's staining methods, demonstrated the iron content of Nissl bodies. He demonstrated the presence of organically bound iron in the nucleolus, the oxychromatin of Heidenhain and in the chromophilic substance of the

cytoplasm (Nissl bodies). He considers the stainable substance of Nissl, for the most part, an iron-containing protein. Scott [15] confirmed the presence of iron in Nissl bodies by microincineration, pointing out, however, the comparatively larger amount of other minerals they contain. Policard [82] had pointed to the fact that nuclei in adults, on the whole, do not contain enough iron to show yellow coloring by microincineration. We can confirm this definitely for the nucleus of the adult ganglion cell.

The glial ground net and the nuclei and processes of glia cells in the gray matter of the spinal cord contain a fair amount of mineral (Figs. 12, 13, 14), more than the white matter (Fig. 2).

The following examples of pathological changes in anterior horn cells are taken from a series of animal experiments done in collaboration with Dr. W. E. Patton. In cats, which have been subjected to experimental dehydration for a series of days, anterior horn cells show severe changes. The globular mineral deposits undergo dusty degeneration (Fig. 15) and, in some instances, deteriorating ganglion cells are clasped by proliferating neuronophagic oligodendroglia and microglia, the nuclei of which are extremely rich in mineral (Fig. 16). In some instances, mineral deposits of affected ganglion cells shift from the globular Nissl bodies into the interglobular spaces that are normally occupied by neurofibrils (Fig. 14). In later stages the centers of the cells become demineralized, while minerals accumulate along the edges of the cells (Fig. 17). The affected cells become rounded out and present a swollen appearance, comparable to that of pellagral cell disease. In later stages the cells become swollen, rounded and completely demineralized (Fig. 18), with the exception of a few granular deposits along the edges of the cells.

The nerve cell changes in senile dementia, especially Alzheimer's and Pick's disease, will be described later.

Normal and Pathological Ependyma in Microincineration

Normal ventricular ependyma (Fig. 21) is rich in minerals contained mostly in the nuclei of ependymal cells. Most of the ash is white and insoluble in water. Partial depletion of cells, as described by Policard, Morin and Nétik [83] for secretory cells in the pancreas, was never observed by us in the ependyma.

In granular ependymitis (in dementia paralytica, and also in Wernicke's superior hemorrhagic encephalitis in chronic alcoholism with beginning delirium tremens), the mineral content of the ependyma increases greatly, especially in the ependymal villi and in the plaques with increased numbers of rows of ependymal cells (Fig. 23).

The finding of abundant mineral deposits in the ependyma is interesting. Schoenholz [45] found in young placentas that the epithelium was richer in minerals than the stroma. Policard and Bonamour [84] found the anterior and posterior epithelium of the cornea much richer in minerals than the cornea itself. Policard [85] found vacuoles in the endoplasm of Vorticellae lined by mineral deposits. Horning and Scott,[86] comparing the two types of holophytic (saprozoic) and holozoic protozoal infusoria, found that in Opalina (a holophytic infusorian) only the dispersed vegetative granules of the endoplasm contained mineral ash, while in Nyctotherus (a holozoic infusorian) the vacuoles of the endoplasm and the oral cavity were lined with mineral deposits.

Amyloid Bodies

Amyloid bodies are rich in minerals and stand out as homogeneous, bright, highly mineral-containing bodies along the edges of the central nervous system (Fig. 20). Most of the ash of amyloid bodies is white, some of it bluish. Here and there are amyloid bodies in which a slight yellowish tinge indicates the presence of traces of iron. Most of the white and yellowish ash is non-soluble in water. Although their iron content is only slight and restricted, it was successfully demonstrated by von Lehoczky,[87] and confirmed by Merritt, Moore and Solomon,[88] in the brains of paretics and non-paretics.

Mineral Structure of Cerebral Blood Vessels

The mineral structure of cerebral blood vessels is one of the most problematic and controversial subjects in the literature concerning microincineration. No completely conclusive description of mineral in the various layers of blood vessels, either normal or pathological, has yet been given. Probably one of the reasons is the difficulty in technique, especially that of irregular shrinkage. Policard and

Ravault [89] recommend boiling blocks for 1 to 2 minutes in 95 per cent alcohol after fixation, but by careful dehydration and embedding we have been getting satisfactory results without this procedure. The other difficulty is one incurred in obtaining complete incineration of extremely tough and heat resistant fibrous tissue. In working on blood vessels one will have to discard many specimens; only carefully cut, even thin sections give reliable pictures. No incompletely incinerated preparation should be used; remaining carbon may severely distort, sometimes almost invert the darkfield picture.

The first description of incinerated blood vessels was given by Ravault.[90] He found the ashes of the vessel wall to be made up of calcium, some magnesium, and possibly very little silica. According to his findings most of the ash is located in the media, while the intima and adventitia contain less. These minerals were found to really impregnate the structures of the media; they are not amorphous mineral deposits as, for instance, those in an atheroma. With great reserve the author identifies the mineral deposits with the elastic lamellae (page 46) and says: "These ashes are arranged in fine stripes oriented parallel to the elastic layers of the aortic wall. One has the impression that the calcium shown by incineration is actually incorporated in the tissue and one cannot keep from superimposing this mineral skeleton to the elastic framework of the mesaorta." However, Ravault warns against making too definite a conclusion, since possibly *intra vitam* this calcareous impregnation may be diffuse and equally distributed over the elastic fibers and interlamellar substance. In a later paper on pathological changes of the vessel wall Ravault [91] found that in calcification of the media precipitation of calcareous substances occurs as the conclusion of profound alterations of the vessel wall, affecting the muscle fibers, elastic layers and ground substance as well. Schultz-Brauns [41] also speaks of an increase of ash in the elastic fibers. Zinkant [46] found increased ash deposits, mostly calcium, in the elastica interna of uterine arteries from multiparous females in the fifth decade of life in incinerated sections 15 μ in thickness, and says that "the accumulation of inorganic substances occurred especially and most markedly in the media, in many instances also especially in the elastic layers of the internal elastic membrane." In contrast to these findings Ham [92] found that the normal aorta of rats contained but little mineral matter. His Figures 1 B and 2 B, from the aorta of a

normal rat and from that of a rat 24 hours after administration of activated ergosterol, show the elastic fibers to be free of mineral. Later, however, in hypervitaminosis D, he saw the elastic fibers also become involved in the process, and on occasion show heavy infiltration with minerals. In other places, however, Ham speaks rather of incrustation on, or along, the elastic fibers. One year later Ku [93] considered the ash of normal coronary arteries to be derived principally from elastic fibers, but also in small part from the intima, media and adventitia. This ash is composed almost entirely of calcium salts. In a 46 year old female (Case 8, Table V), however, he describes two kinds of ash: one faintly illuminated, located in the fibrous parts of the intima; the other brightly illuminated, located in the elastica.

In spectroscopic studies Policard, Morel and Ravault [94] found calcium and magnesium in normal and pathological blood vessels, thus confirming earlier chemical work of Baldauf.[95] From their detailed studies they concluded that normal intima contains more calcium than magnesium. The media contains more calcium and much more magnesium than the intima. In lipoid degeneration of the intima calcium increases, but not magnesium. In the media underlying atheromatous plaques both calcium and magnesium are diminished. In calcified atheromata calcium is increased but not magnesium. Pathological mineral deposits in the aorta are always calcareous, the magnesium does not participate in this process but, on the contrary, tends to diminish in these lesions. The authors do not give data on the elastica and in a review in Cowdry's handbook on arteriosclerosis Policard [96] gives none.

In our studies we found most of the mineral of the vascular wall in collagenous fibrous tissue, less in muscle, and still less in reticulin, while elastic tissue was always found to be free of mineral residue. Most of the mineral deposits are insoluble in water, white, and only slight bluish deposits are present. The deposits are richest in the media, but are somewhat less in amount in the intima and adventitia. The complete absence of mineral in elastic tissue is of interest. So far only two other tissues have been found to be free of mineral, namely the axis cylinders of the nervous system (Scott,[15] confirmed by our studies) and the isotropic J bands of striated muscle (Scott [97]).

The wall of a normal meningeal artery contains a considerable

amount of mineral in all layers except the elastica interna, which stands out as a dark, winding narrow band, free of mineral ash (Fig. 22).

In various diseases of the vessel wall, such as arteriosclerosis with proliferation or hyalinization of the intima, hyalinization of the media, or syphilitic endarteritis, the internal elastic membrane always retains its character as an ash-free structure.

Figure 24 shows an oblique longitudinal section through a larger branch of the arteria chorioidea, taken from its extracerebral course near the Ammon's horn from a case of cerebral arteriosclerosis (a 56 year old male). The mineral residue is most dense in the media, while the newly formed intimal proliferation contains somewhat less. The inner elastic membrane, although in some places delaminated, can still be seen as a dark, non-mineral-containing layer, which in places of delamination is split into two or three equally dark, ash-free layers. In between the layers mineral-containing, newly formed collagenous fibrous tissue can be seen. In many places the mineral-free, inner elastic membrane, especially its inner lamina, is seen to be perforated by many fine, mineral-containing collagenous fibers which connect the inner intimal proliferation with the outer part of the intima and the media. This confirms Ranke's[98] findings of fenestrations of the elastic membranes, and especially his findings concerning the increase of these fenestrations in arteriosclerosis and related vascular diseases. In thrombosed vessels the fine network of fibrin, and later the network of reticulin, contains very little mineral residue (Fig. 27, upper half of picture). The nuclei of the emigrating fibroblasts and endothelial cells, however, the newformed collagenous fibrous tissue, and especially the lining of recanalized tissue, is richer in minerals (Fig. 27, left lower quarter of figure). Most rich in mineral, however, are the large siderophagic scavenger cells, which take up the hematogenous iron from the clot and stand out by reason of considerable amounts of ingested dark yellow and red iron oxide (Fig. 27, right lower quarter of figure, near the wall of the vessel). These cells frequently are found also to contain lipoid, as found by us in collaboration with Dr. T. J. Putnam, and thereby resemble Ciaccio cells (Baginski[99]).

Mineral Structure of Vascular Lesions of the Brain

Mineral Residue of Hemorrhages, with Reference to the Mineral Content of Various Elements of the Blood

Staining methods do not reveal the iron of hemoglobin (Macallum [100]). Policard [33, 35, 101] and Policard and Pillet [102] found that although the single red cell does not leave enough ash of sufficient density to be sure of its iron content, agglomerated masses of cells from a fresh smear or in fluid blood in blood vessels leave after incineration a definitely yellowish, iron-containing ash.

In our experience with blood smears two elements stand out by their massive mineral content — the white blood cells and the blood platelets (Fig. 32). The white cells, leukocytes as well as lymphocytes, show dense white mineral deposits in their nuclei; the leukocytes relatively more than the lymphocytes. The cytoplasm of the polymorphonuclear leukocytes shows only a few, fine, faintly stainable bluish granules. According to Scott [15] the eosinophiles may be recognized by white cytoplasmic ash granules. Blood platelets are conspicuous by their particularly dense, brilliant, water insoluble ash. The red cells contain only fine, faintly visible mineral granules, most of them accumulated along the edges of the red cells which thereby, in many instances, appear as rings in the darkfield (Fig. 32). Most of these granules are of a bluish color; only here and there are fine yellowish granules visible. Figure 19 is taken from a case of myeloid leukemia and illustrates the high mineral content in the nuclei of myeloblasts, myelocytes and leukocytes. A smaller amount of mineral is seen in the cytoplasm of these cells. In Figure 19, surrounding the large myeloblast, many blood platelets are seen which are clearly composed of small, regularly distributed mineral granules corresponding to azurophilic granules, a fact that had been observed by Scott.[15]

Perivascular hemorrhages in the brain are conspicuous by their content of yellowish iron-containing mineral (Fig. 25); the yellow color increases in depth as the chemical transformation of hemoglobin into hematoidin and hemosiderin proceeds; by the time of ingestion by scavenger cells the color has changed to orange or deep red.

Mineral Structure of Softening and of Ischemic Necrosis

Foci of softening and ischemic necrosis of the brain stand out as grossly demineralized areas in microincinerated preparations. In the focus of softening (Fig. 26) only scavenger cells and remaining vascular network in the form of engorged, medium sized blood vessels can be seen. The scavenger cells especially stand out as isolated bodies of high mineral content in an otherwise completely demineralized field. Foci of ischemic necrosis show all gradations from complete demineralization of nervous tissue up to a marked or slight diminution of minerals in a more or less sharply circumscribed area. The time required for this demineralization in ischemic necrosis is surprisingly short; we have seen it in lesions of only 1 days duration. The manner in which mineral is removed cannot be determined.

This demineralization in vascular lesions of nervous tissue has certain parallels in bone and in the placenta. Policard,[103] and Policard and Péhu [104] found that during the process of ossification demineralization of the ground substances of cartilage takes place in regions devoid of blood vessels. The authors wonder if this possibly may be due to edema. Schultz-Brauns and Schoenholz,[44] who found such demineralization in the placenta, thought it due to the fact that easily soluble salts, after the death of the cells, are immediately dissolved and removed, the body as a whole being undersaturated in soluble salts. A fully satisfactory explanation, however, has not yet been given.

Mineral in Tumors of the Brain

Policard and Doubrow[9] were impressed by the fact that tumor tissue, by the microincineration process, remained carbonized much longer than normal, but considered it, however, on the whole to be rather poor in mineral ash. Scott and Horning [105, 106] discovered the important fact that tumor tissue, when compared with similar normal tissue, contains a relatively much greater amount of mineral ash, especially of calcium and iron oxide. The nuclei especially contain more ash than the nuclei of normal tissue. The nuclear ash contains also more iron oxide than normal cells, but the cytoplasmic ash is more abundant. In all these features of distribution and arrangement of mineral salts malignant tumor tissue resembles that of the

developing chick embryo (Horning and Scott [79]). The authors see in this a contribution to Cohnheim's embryonal theory of tumor proliferation. In this connection it is of great interest to remember that the water content of tumor tissue, as well as that of embryonic, is higher than that of normal tissue (Schlottman and Rubenow [107]). The water content of pig embryos increases up to 160 mm. length, then gradually decreases until birth (Wilkerson and Gortner [108]).

We are able fully to confirm Horning's and Scott's findings on our material of tumors of the brain, gliomatous as well as metastatic. Even at low power tumors in incinerated preparations can be seen at once to be extremely rich in mineral-containing substances, in contrast to normal brain tissue (Fig. 30). The mineral accumulates especially in nuclei, less in the cytoplasm of tumor cells (Fig. 31). Most of the mineral granules appear white or yellow, fewer are bluish in darkfield; most of the white calcium-containing and yellow iron-containing minerals are insoluble in water. The mineral content of the tumors may possibly play a rôle in their X-ray sensitivity.

Minerals in Inflammatory Lesions of the Brain

Purulent Meningitis and Meningoencephalitis

In purulent meningitis the meningeal exudate is extremely rich in minerals, in contrast with which the brain appears rather dark and poor in minerals (Fig. 28). The mineral of the exudate is mainly accumulated in the nuclei, less in the cytoplasm of polymorphonuclear leukocytes and meningeal histiocytes, and in the nuclei of lymphocytes. Most of the mineral residue is whitish and insoluble in water, less of it bluish and soluble in water. Similar mineral deposits are found in the polymorphonuclear and histiocytic perivascular infiltrations around intracerebral cortical blood vessels in lesions of purulent meningoencephalitis (Fig. 29).

Syphilitic Meningoencephalitis, Gumma and Dementia Paralytica

The inflammatory lesions of syphilitic meningoencephalitis, gumma and dementia paralytica stand out by their extremely rich mineral deposits; the degenerative lesions of dementia paralytica, however, as well as the areas of infarction in syphilitic meningoencephalitis, are characterized by demineralization of nervous tissue.

Iron occurs in microincinerated preparations of neurosyphilitic lesions in two forms: (1) concentrated as dark yellow, orange or red deposits of granules of iron oxide in (*a*) histiocytic cells of meningeal infiltrations, especially around blood vessels; (*b*) histiocytic and microglial elements of intracerebral perivascular cuffs, especially in abundance near gummas; (*c*) isolated perivascular histiocytes and microglial cells throughout the brain, especially the gray matter; and (*d*) microglia cells in focal lesions. These iron deposits do not consist of hemosiderin and are invisible in non-incinerated or unstained preparations. (2) It is also found as an admixture to other minerals as light yellow, granular mineral deposits, indicative of the presence of iron oxide in (*a*) gummas, in the remains of the nuclei of many of the infiltrative cells as well as in the ground substance of the gumma; (*b*) nuclei and cytoplasm of certain plasma cells in perivascular infiltrative cuffs; (*c*) nuclei of certain lymphocytes of perivascular infiltrative cuffs; and (*d*) nuclei and processes of microglia cells in and around lesions.

Most of the infiltrating lymphocytes and plasma cells are free of iron, as are all macroglial astrocytes, including those that show active growth and proliferation in and around lesions. These cells contain only whitish and bluish ash. This ash is contained in the nuclei of lymphocytes, plasma and macroglial cells. It is also seen in the cytoplasm of these cells but not in abundance except in the cytoplasm of the proliferating astrocytes, which contain a great deal of ash.

Perivascular syphilitic infiltrations, especially in the region of gummas, show the difference in iron content between the various infiltrative elements most impressively. Some of these infiltrations show heavy, dark colored iron deposits only at one particular point at the periphery of the perivascular infiltration. Stained control sections reveal that these deposits are exclusively contained in histiocytic and microglial elements which accumulate at this point. The centers of such dense perivascular infiltrations reveal only faintly yellowish granules, indicating traces of iron oxide in the nuclei and cytoplasm of a few plasma cells and in the nuclei of a few lymphocytes. The majority of the lymphocytic and plasma cell infiltrations contains whitish and bluish mineral granules in the nuclei and in the cytoplasm of the latter.

Gummas are among the most highly hypermineralized lesions of

the nervous system. At low power (Fig. 33) they stand out as light glistening areas, owing to their extremely rich mineral content. The mineral within the gumma is also much denser in its distribution than the mineral in normal tissue or even in tumor tissue; its structure, however, is not quite homogeneous, but definitely granular. At higher power the gumma appears to be composed of larger and smaller granules, arranged somewhat regularly. The larger granules are composed of dense aggregates of small, yellowish and white granules corresponding to remains of nuclei and nuclear fragments of the original gummatous infiltration. The small granules are rather evenly distributed in between and are composed of light yellowish, whitish and bluish granules: in some places yellowish ashes, in others bluish predominate. In addition to these granules the incinerated preparations reveal also fine mineralized fibers, most of them toward the edges of the gumma. Some of them are primitive, non-collagenous fibrous tissue fibers, others are processes of microglia cells, which can be seen in active proliferation at many places along the edges of the gumma. Ramified microglial elements can be identified. Also, a few macroglial astrocytes are seen here, recognizable by their plumper shape and exclusively white and bluish mineral content. We have not yet been able to identify spirochetes in microincinerated preparations.

Minerals in Multiple Sclerosis

The plaques of multiple sclerosis are demineralized lesions. In microincinerated preparations at low power, they stand out as dark areas, owing to the fact that their ground substance has become void of mineral salts (Figs. 34, 35, 36, 37). The engorged blood vessels of early hyperemic plaques, however, stand out as brightly illuminated spots (Figs. 37, 40), and the meshes of the glial network and macroglial astrocytes are well outlined as light, mineral-containing bodies against the black background of the plaque (Figs. 39, 40).

The engorged small blood vessels and capillaries, many of which contain early thrombi, show in their adventitial spaces, in some instances, in addition to lymphocytic-histiocytic infiltration, large phagocytic cells that are rich in reddish yellow granules of iron oxide (Figs. 38, 40). Many of these same cells, as shown by control slides,

also contain lipoid material. The admixture of minerals with lipoids, which also occurs in Ciaccio cells (Baginski [99]), may furnish part of the crystalline appearance of cholesterol esters, described by Amorim [109] in phagocytic cells around blood vessels in lesions of the nervous system.

Especially impressive is the appearance of macroglial protoplasmic astrocytes in microincinerated preparations of these lesions (Figs. 39, 40). Their nuclei, cell bodies and processes with all their ramifications, are well outlined by a rich mineral content. The nuclei, however, contain only slightly more or less mineral than the cell bodies and, therefore, in many instances differentiation is not clear (Fig. 40). The minerals consist of dense, whitish and bluish granules, the former being in the majority. Most of the white granules are insoluble in water and do not contain iron oxide. This picture presented by macroglial astrocytes in incinerated preparations in its completeness compares well with specimens prepared by Cajal's gold sublimate method.

In the older anemic plaques, which stand out as grossly demineralized areas (Fig. 41), many smaller blood vessels have undergone obliteration, while on the demineralized background of the plaque, well outlined, large, mineral-containing fibrillary astrocytes can be seen (Figs. 41, 42). These findings prove that demineralization is an additional, essential feature in the pathology of these lesions which have customarily been referred to as demyelinizing. This term, however, takes into account only one part of the total picture, which is characterized by demineralization as well.

It is of great interest to find that the plaques in multiple sclerosis in their mineral architecture most closely resemble foci of softening or of ischemic necrosis, all these being essentially lesions of demineralization. These findings tend to confirm the observations of Putnam,[110, 111] who described the multiple sclerotic plaques as regressive lesions of vascular origin.

Minerals in Senile Dementia

Pick's and Alzheimer's Disease

In the cortical disease of senile dementia loss and damage of ganglion cells, disarrangement of cortical architecture and gliosis are well recognizable in incinerated preparations. Areas depleted of

ganglion cells appear also demineralized by their loss. The alteration in shape of ganglion cells is well recognizable, also the condensation of their mineral deposits in shrinkage (Fig. 48). These mineral deposits are white, most of them insoluble in water. The nuclei of glia cells, and to less extent their cytoplasm and processes, are well outlined by their mineral content; places of intense gliosis show at low power a local increase of mineral. The small glia rosettes which clasp or replace necrotic ganglion cells (Fig. 48) are well recognizable by the dense mineral content present in the nuclei and in a few of the processes of the small groups of oligodendroglial and microglial cells that make up these rosettes. Most of their mineral content is white and insoluble in water, less of it bluish.

Figure 47 shows an incinerated section from the area entorhinalis of a case of Pick's disease in a 72 year old female. The pathology of this area in diseases of the cerebral cortex, including Pick's disease, has recently been described by Rose.[112] We are able to confirm Rose's findings in our microincinerated preparations. The disturbance of cortical architecture is obvious at low power (Fig. 47). Most conspicuous is the almost complete loss of ganglion cells in the outer part of the lamina principalis externa (Pre-*Alpha*); somewhat less complete is the loss of ganglion cells in the lamina principalis interna (Pri), while in the inner part of the lamina principalis externa (Pre-*Beta* and *Gamma*) and in the lamina dissecans (Ds) a great many ganglion cells are preserved. Many of them, however, show pathological changes such as shrinkage with condensation of mineral matter, corkscrew deformity of processes and in some instances complete pyknosis. These layers show also considerable gliosis, especially of microglial, but also of oligodendroglial cells. Many of the processes of microglial cells are faintly visible, even at this low power, owing to their content of whitish, bluish and yellowish mineral deposits.

The senile plaques in senile dementia, and especially in Alzheimer's disease, when subjected to microincineration burn out completely and do not leave any appreciable traces; their site is neither marked by excess mineral residue, nor by demineralization. This is well illustrated by Figures 43 and 44, which are neighboring sections from a series of the cornu ammonis (field H 4 and fascia dentata) from a case of Alzheimer's disease in a 74 year old female. Figure 43 is stained with a modification of Bielschowsky's silver impregnation

for paraffin sections; Figure 44 is microincinerated. The cellular ribbon of the fascia dentata in the lower half, and the blood vessel in the left lower corner of each picture are landmarks that make orientation easy. In the Bielschowsky stained Figure 43 three senile plaques are well shown: one is adjacent to the dorsal surface of the fascia dentata, and two are in the upper half of the picture in field H 4. In the microincinerated neighboring preparation (Figure 44) no trace of these plaques is visible at low power; they do not stand out either as areas of increase or of decrease of tissue minerals. Figures 45 and 46 show one of these plaques, the one adjacent to the fascia dentata, at higher power. In Figure 45, a Bielschowsky stained preparation, the plaque appears clearly, dorsad from the cellular ribbon of the fascia dentata, and to the left of a small blood vessel. In Figure 46, a microincinerated preparation, the cellular ribbon of the fascia dentata, the small blood vessel and the other landmarks of the tissue are equally well demonstrated but the senile plaque is at first invisible. On closer examination, however, the plaque can be identified to the left of the small blood vessel as a slight disarrangement of the glial reticulum which, however, is neither poorer nor richer in minerals than the rest of the glial ground net of the tissue. These findings tend to disprove von Braunmühl's [113] theory, which considers the senile plaques inorganic mineral precipitates and, to some extent, favor Bouman's [114] theory, which considers them to be metaplasias of the glial reticulum. We conclude from our studies that senile plaques are of proteid or other organic material and do not contain mineral deposits because they burn out on microincinerated slides. Their exact nature has not yet been determined. Hechst [115] and Winkler-Junius,[116] with reference to the work of Bennhold,[117] Divry,[118] and Leupold,[119] stressed the amyloid content of the plaques, which Winkler-Junius considered amyloid degeneration of phagocytic microglia cells, thought to make up the centers of plaques. Recently Gerstmann, Sträussler and Scheinker [120] described atypical formations, closely resembling senile plaques, in a 28 year old patient suffering from a heredodegenerative spinocerebellar ataxia with cortical involvement. However, these formations, although distinguished by increased argentophilia, similar to senile plaques, are stained vividly also with various other stains.

The neurofibrillar strands in Alzheimer's ganglion cell disease,

however, are well demonstrated in microincinerated slides, standing out as amply mineral-containing interlaced strands, while the remainder of the cytoplasm of the cell is demineralized (Fig. 49). The mineral deposits in Alzheimer's neurofibrillar strands are white and for the most part non-soluble in water. Figure 50 shows an interesting group of three ganglion cells from the subiculum of the same case. In the left half of the picture a characteristic Alzheimer cell is seen with, however, only marginal arrangement of the thickened hypermineralized strands. The upper cell in the right half of the picture is about normal; its granuloglobular mineral deposits correspond to the Nissl bodies. The third cell, in the lower part of the right half of the picture, appears normal only in its upper apical part, above the nucleus, while in its basal part the cytoplasm is filled with thickened hypermineralized Alzheimer strands. Bielschowsky [121] was the first to describe pathological cells in Alzheimer's disease which showed Alzheimer strands only in their basal part, near the pigmented spot, while the rest of the cell showed normal neurofibrillar structure. We are able to confirm this finding for the mineral structure also of these cells. Bielschowsky [122] deduced from this finding that Alzheimer's strands are the result of impregnation of neurofibrillar structures with foreign substances. We are able to conclude from our microincineration studies that one of these substances is mineral, since normal neurofibrils are free of mineral, while Alzheimer's neurofibrillar strands contain mineral. The mineral deposits alone, however, cannot be the cause of the argentophilia of Alzheimer's neurofibrillar strands because the senile plaques, although equally argentophilic, are free of mineral. Von Braunmühl [113] considers Alzheimer's strands to be the result of hydration and swelling of previously dehydrated structures. One of us,[123] in collaboration with Wu,[124] has demonstrated that thickened, argentophilic neurofibrillar strands are found in cortical ganglion cells of patients who died from dysentery, cholera or intestinal tuberculosis in a cachectic, emaciated and dehydrated condition and whose brains showed pseudoatrophy and gave evidence of an altered water-binding capacity. It was also shown that somewhat similar, thickened argentophilic neurofibrillar strands could be produced artificially in fresh brain tissue exposed to water or saline solutions of various concentration. To these indications of an altered, possibly relatively increased water content in Alzheimer strands we can

add the new fact of an increased mineral content, clearly demonstrated by microincinerated preparations. The simultaneous increase of both water and mineral content is not incompatible, since in tumor and in embryonic tissue, water as well as minerals is found to be increased; while in ganglion cell disease produced by experimental dehydration, demineralization was observed. These observations, although throwing some light on this condition, do not yet offer a conclusive explanation.

The main conclusions drawn from our data on the microincineration of senile plaques and Alzheimer's neurofibrillar strands are: (1) Alzheimer's neurofibrillar strands in microincinerated preparations differ from normal neurofibrils in that they contain mineral deposits, while normal neurofibrils contain none. (2) Senile plaques and Alzheimer's neurofibrillar strands, which are usually regarded as pathological changes of similar type (Bouman [114]) on the basis of their argentophilia, differ fundamentally in microincinerated preparations as senile plaques are free of mineral, while Alzheimer's neurofibrillar strands contain mineral deposits.

Summary and Conclusions

1. The cerebral cortex of the newborn human infant is rich in minerals (calcium, iron, and certain water soluble minerals). Most of it is contained in the nuclei of the ganglion cells and glial elements of all layers, while the cytoplasm of these cells contains very little or no mineral ash.

2. In the normal adult the ganglion cells of pyramidal type in the cerebral cortex, the Purkinje cells in the cerebellum, and the anterior horn cells in the spinal cord contain considerable mineral deposit in their nucleolus, cytoplasm and dendrites. The nucleus is essentially free from mineral residue except for a few fine, faintly visible strands, especially along the nuclear membrane. The mineral deposits in the cytoplasm are globular and granular in character and correspond in their arrangement to the Nissl bodies. They contain calcium, very little iron oxide, and certain water soluble minerals. The intracellular neurofibrils, axone hillock and axis cylinder are free from mineral ashes.

The granular cells of the cerebellum contain more mineral residue than those of the cerebrum.

The glial ground net, nuclei, and certain processes of glial cells contain a fair mount of mineral, most of it white and for the most part insoluble in water.

3. Dusty deterioration, homogenization and destruction of the Nissl substance in diseased ganglion cells is demonstrated in microincinerated preparations to be associated with severe changes of the mineral structure of the affected ganglion cells and ranges from dusty deterioration and shifting of the minerals to complete demineralization.

4. The ventricular ependyma is rich in minerals, most of its ash is white and for the most part insoluble in water. The mineral content of the ependyma increases greatly in granular ependymitis.

5. Amyloid bodies are rich in minerals, especially in calcium; traces of iron are found only at times.

6. The walls of the cerebral blood vessels are rich in white mineral ash which for the most part is insoluble in water. Most of these mineral deposits are found in the collagenous fibrous tissue, less in the muscle tissue, still less in the reticulin, while the elastic tissue was always found to be free from mineral residue. Also, in various diseases of the vessel wall, such as arteriosclerosis with proliferation or hyalinization of the intima, hyalinization of the media, or syphilitic endarteritis, the internal elastic membrane always retains its character as an ash-free structure. Thereby the fenestrations and perforations of the elastic membranes and their increase in arteriosclerosis and related vascular disease stand out conspicuously in microincinerated preparations. In thrombi of various ages fibrin and reticulin contain very little mineral residue; the nuclei of emigrating fibroblasts and endothelial cells, however, as well as the new formed collagenous tissue and the lining of recanalizations, are richer in mineral. Most rich in mineral (most of it iron oxide) are the large siderophagic scavenger cells in and around thrombosed vessels.

7. Cerebral hemorrhages are conspicuous by their content of yellowish iron-containing mineral. The yellow color increases in depth and may change to orange or deep red as the chemical transformation of the hemoglobin into hematoidin and hemosiderin proceeds.

The mineral content of the various elements of the blood is discussed.

8. Foci of softening and ischemic necrosis are characterized by demineralization. In the former only the scavenger cells and a few

blood vessels stand out as isolated mineral-containing bodies in an otherwise completely demineralized field.

9. Gliomatous and metastatic brain tumors are outstanding as areas of hypermineralization. The mineral accumulates in excess in the nuclei, less in the cytoplasm of the tumor cells.

10. Inflammatory lesions of the nervous system, including those of purulent meningitis, purulent meningoencephalitis, syphilitic meningoencephalitis, gumma and dementia paralytica are characterized by hypermineralization. The mineral is accumulated in the nuclei, in the nuclear fragments, and in the remains of the infiltrative cells, as well as in the cytoplasm and the processes of certain of these cells, especially in those of glial origin. The iron content of certain infiltrative cells in neurosyphilis is demonstrated well in microincinerated preparations.

11. The lesions of multiple sclerosis are characterized by demineralization. Only the engorged and thrombosed blood vessels, the infiltrative cells, including iron-loaded scavenger cells, the macroglial astrocytes and the meshes of the glial reticulum, stand out as the sole remaining mineral-containing bodies in an otherwise completely demineralized area.

12. Areas and layers in senile cortical atrophy, and especially in Pick's disease, which are depleted of ganglion cells appear demineralized by their loss.

13. Senile plaques in senile dementia, and especially in Alzheimer's disease, when subjected to microincineration burn out completely. Their site is neither marked by excess mineral residue nor by demineralization. The only trace of them recognizable in microincinerated preparations is a slight disarrangement of the glial reticulum which, however, is neither poorer nor richer in ash than the rest of the glial ground net of the tissue.

14. The neurofibrillar strands in Alzheimer's ganglion cell disease differ from normal neurofibrils: Alzheimer's strands contain ample mineral deposits, while normal neurofibrils are free from mineral ash. The mineral deposits in Alzheimer's neurofibrillar strands are white and for the most part insoluble in water. The remainder of the cytoplasm of these cells is demineralized.

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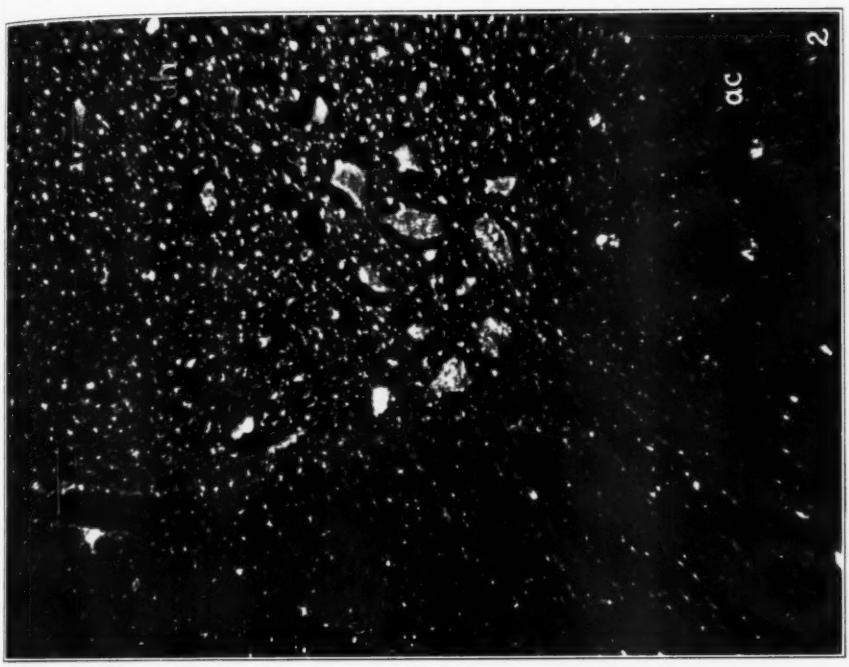
DESCRIPTION OF PLATES

Unless otherwise stated all sections were cut at 4 μ, subjected to the microinceration process, and examined by darkfield illumination.

PLATE 63

FIG. 1. Newborn premature human infant. Cerebral cortex, area 10, third layer. × 530.

FIG. 2. Anterior horn (ah) and anterolateral column (ac) from a normal spinal cord. Cat. × 156.



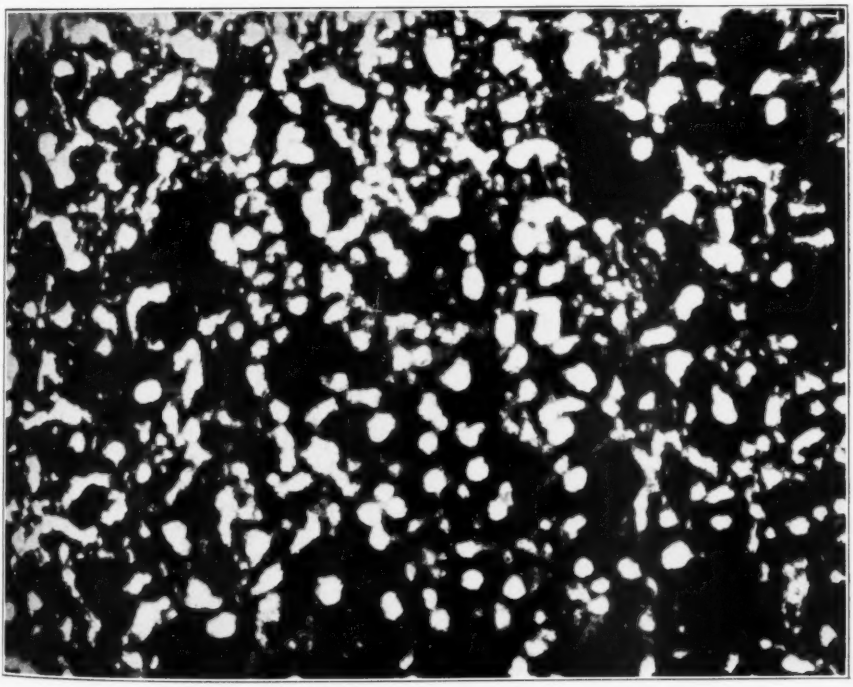


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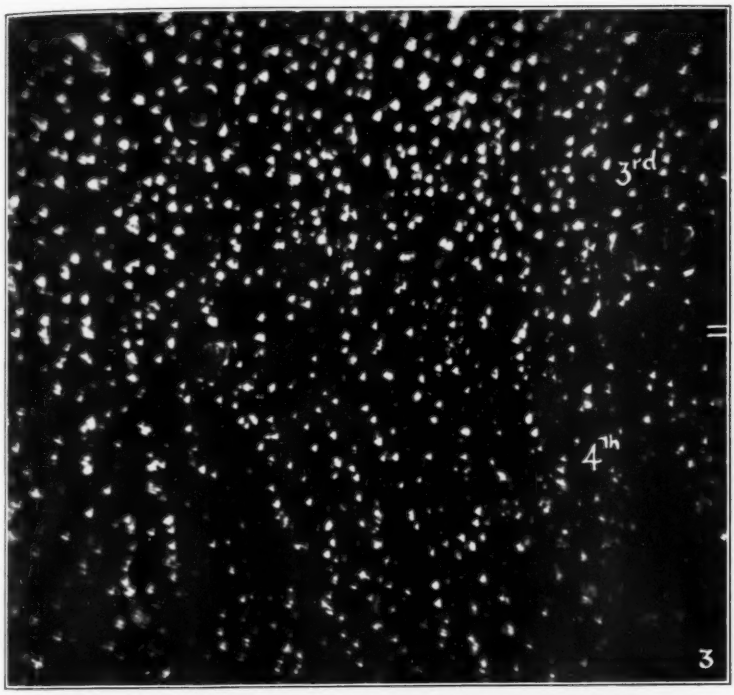
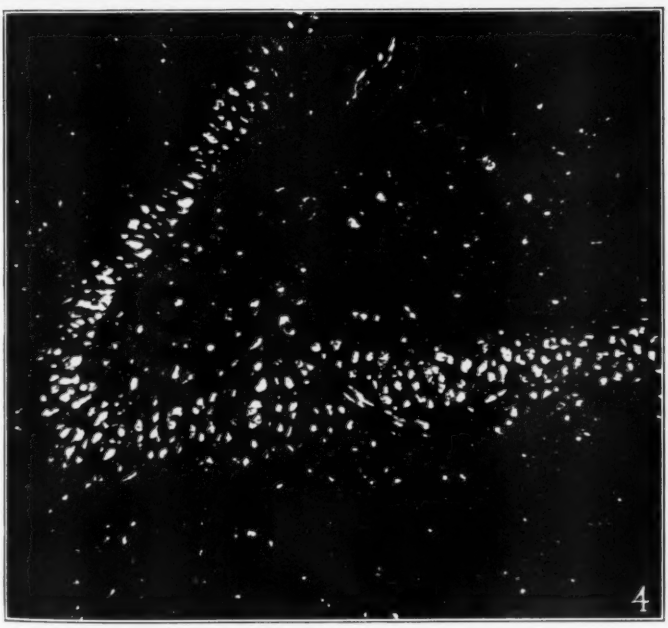
PLATE 64

FIG. 3. Normal adult male. Cerebral cortex, area 10, third and fourth layer. × 100.

FIG. 4. The fascia dentata of the cornu ammonis of a normal human adult. × 125.





Alexander and Myerson Mineral Content of Cerebral Lesions

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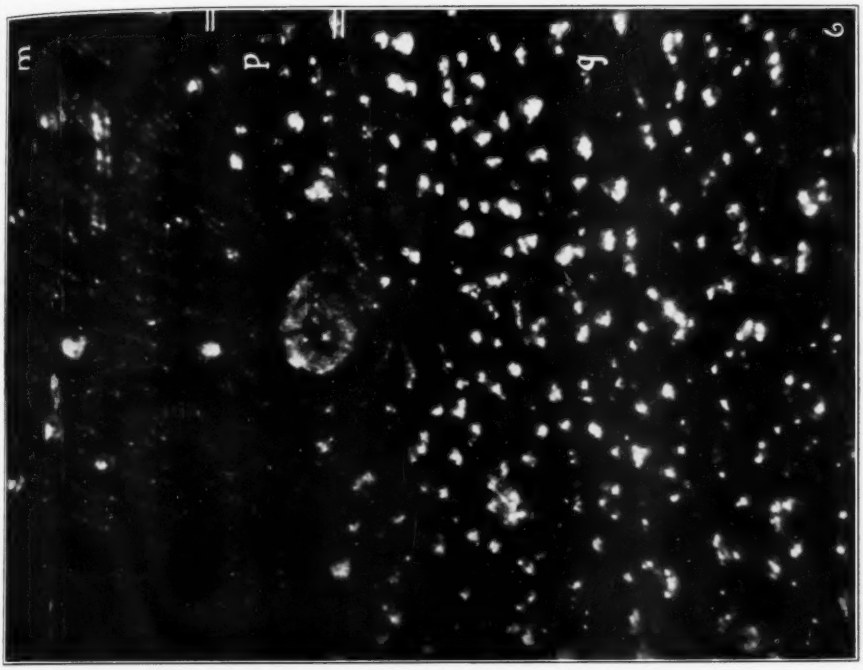
PLATE 65

FIG. 5. The cerebellar cortex of a normal human adult. × 110.

FIG. 6. Normal human adult. Cerebellar cortex. m = molecular layer; P = Purkinje layer; g = granular layer. × 576.



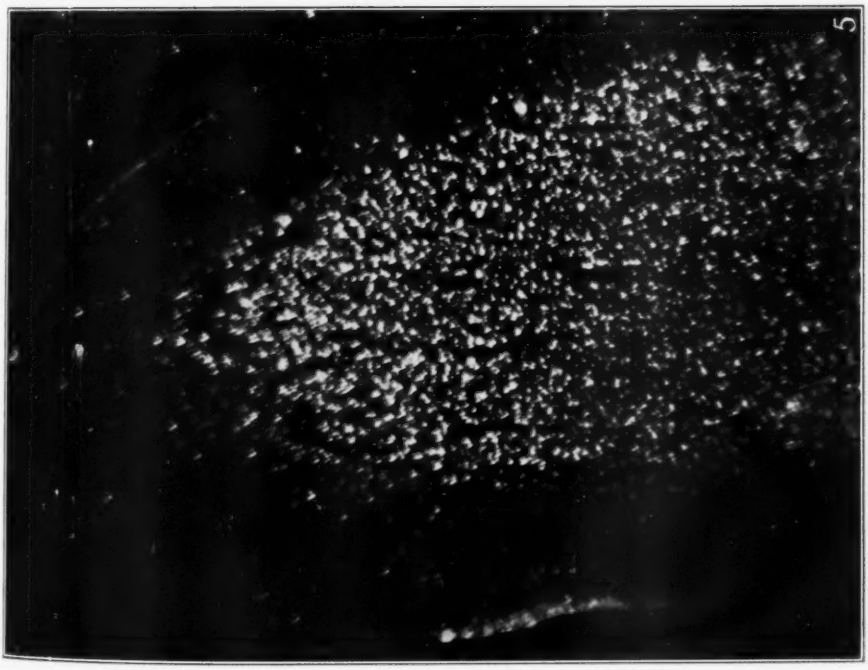


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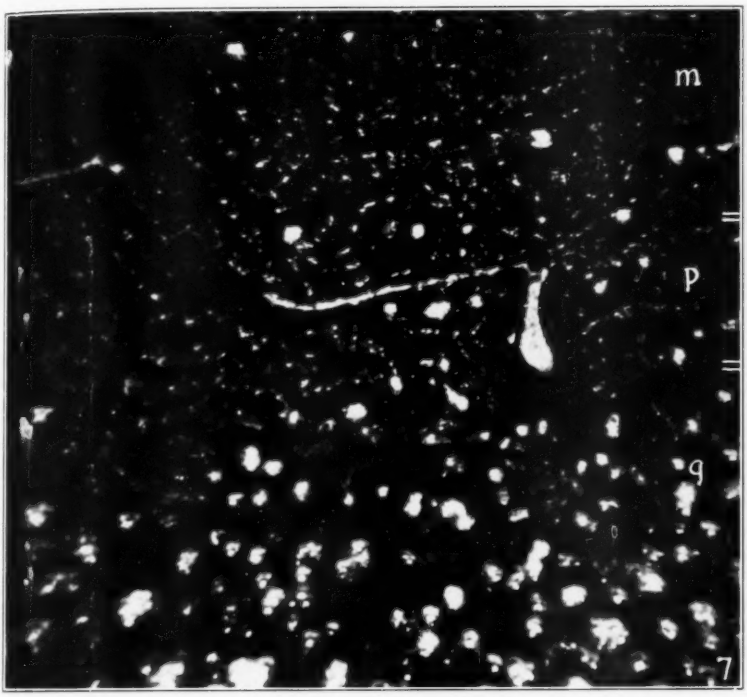
PLATE 66

FIG. 7. Normal human adult. Cerebellar cortex. m = molecular layer; P = Purkinje layer; g = granular layer. × 576.

FIG. 8. Normal human adult. Cerebellar cortex. C = Cajal's basket. × 576.



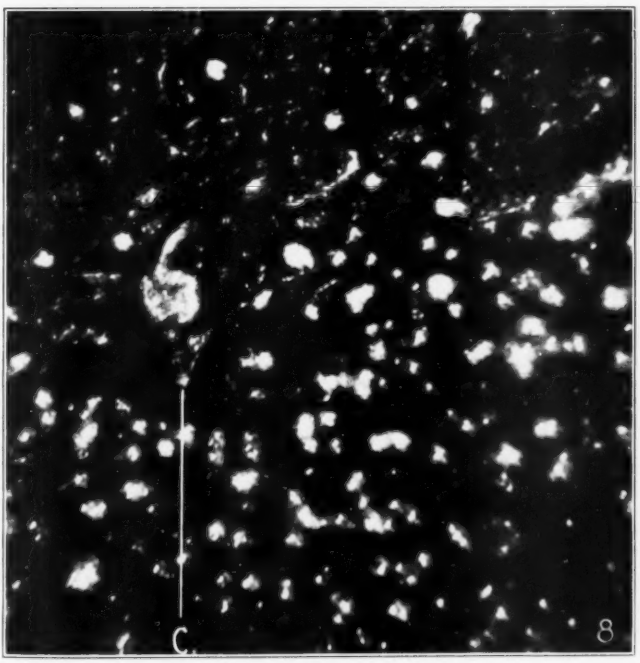


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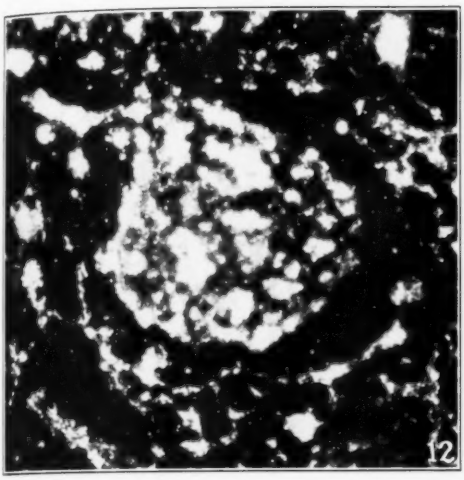
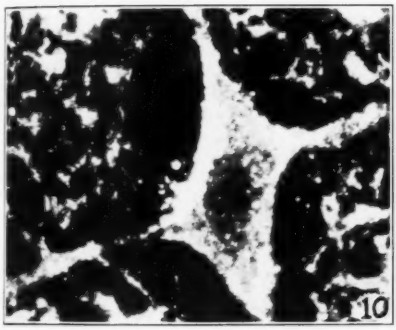
PLATE 67

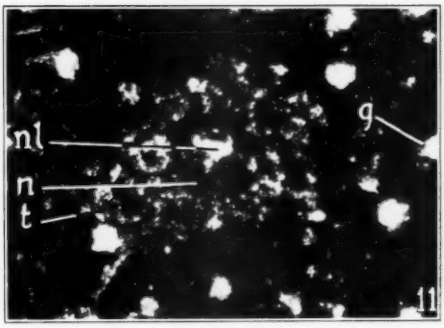
FIG. 9. Normal pyramidal ganglion cell from the cornu ammonis (field H 2). Human adult. × 760.

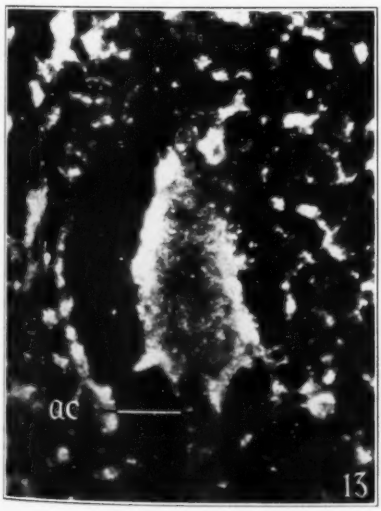
FIGS. 10, 11, 12 and 13. Normal ganglion cells from the anterior horn of the spinal cord of a cat. n = nucleus; nl = nucleolus; t = tigroid bodies; g = glia cell; ac = axis cylinder. × 760.

FIG. 14. Cat, experimental dehydration. Ganglion cell from the anterior horn of the spinal cord. Mineral deposits are present in interglobular spaces. × 760.









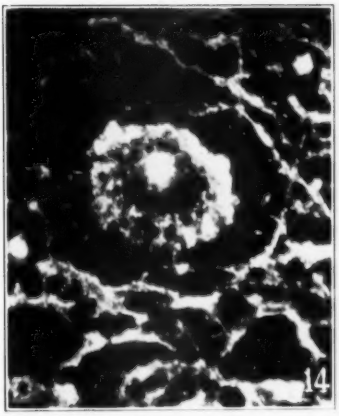


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PLATE 68

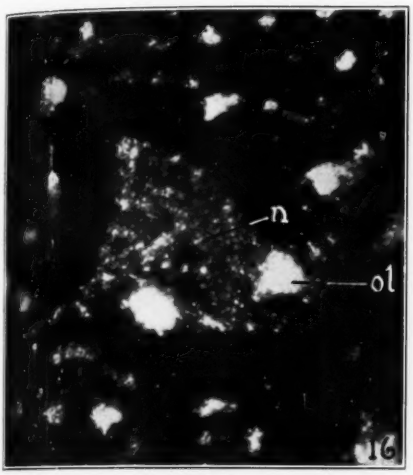
FIG. 15. Cat, experimental dehydration. Ganglion cell from the anterior horn of the spinal cord. Dusty deterioration of mineral deposits corresponding to tigroid substance of cytoplasm. × 760.

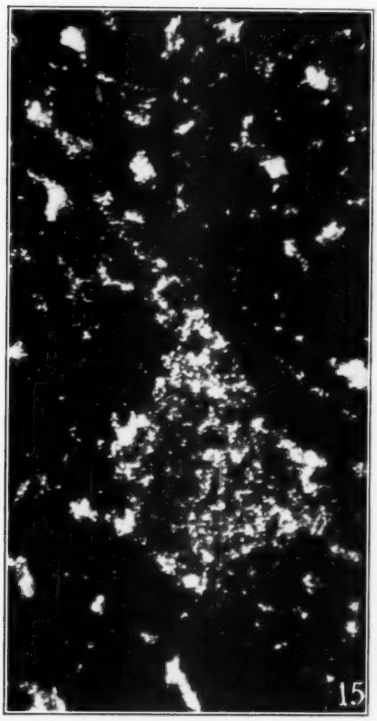
FIG. 16. Cat, experimental dehydration. Ganglion cell (n) from the anterior horn of the spinal cord. Dusty deterioration of mineral deposits, corresponding to tigroid substance of cytoplasm. Cell clasped by oligodendroglia cells (ol). × 760.

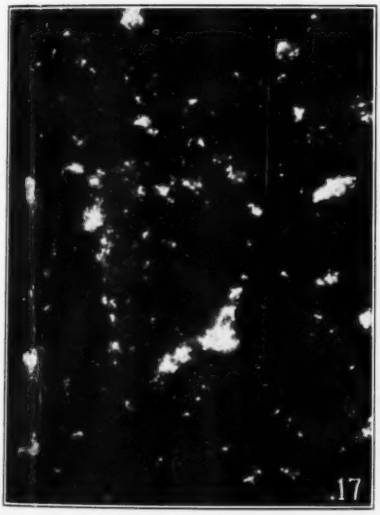
FIG. 17. Cat, experimental dehydration. Ganglion cell from the anterior horn of the spinal cord. Central demineralization. × 760.

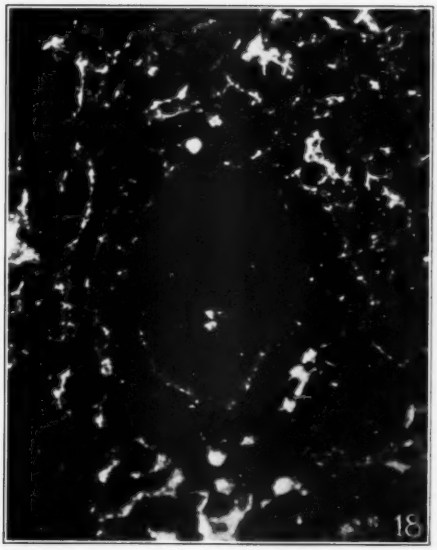
FIG. 18. Cat, experimental dehydration. Ganglion cell from the anterior horn of the spinal cord. Complete demineralization. × 760.

FIG. 19. A myeloblast and several thrombocytes from a blood smear from a case of myeloid leukemia. × 800.









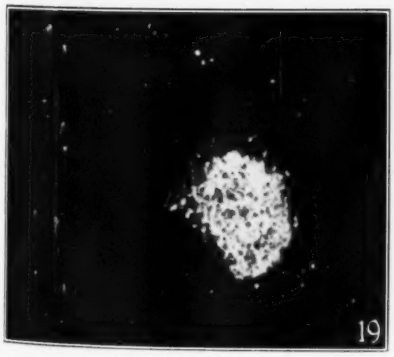


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PLATE 69

FIG. 20. Amyloid bodies in the marginal glial layer of the gyrus hippocampi of a 56 year old male with cerebral and generalized arteriosclerosis. am = marginal glial layer, containing amyloid bodies; s = subarachnoid space. × 115.

FIG. 21. Normal adult male. Ependyma (e) and subependymal tissue (s) of the wall of the third (III) ventricle. × 110.

FIG. 22. Normal extracerebral (meningeal) artery. Branch of the arteria chorioidea. Human adult. × 115.

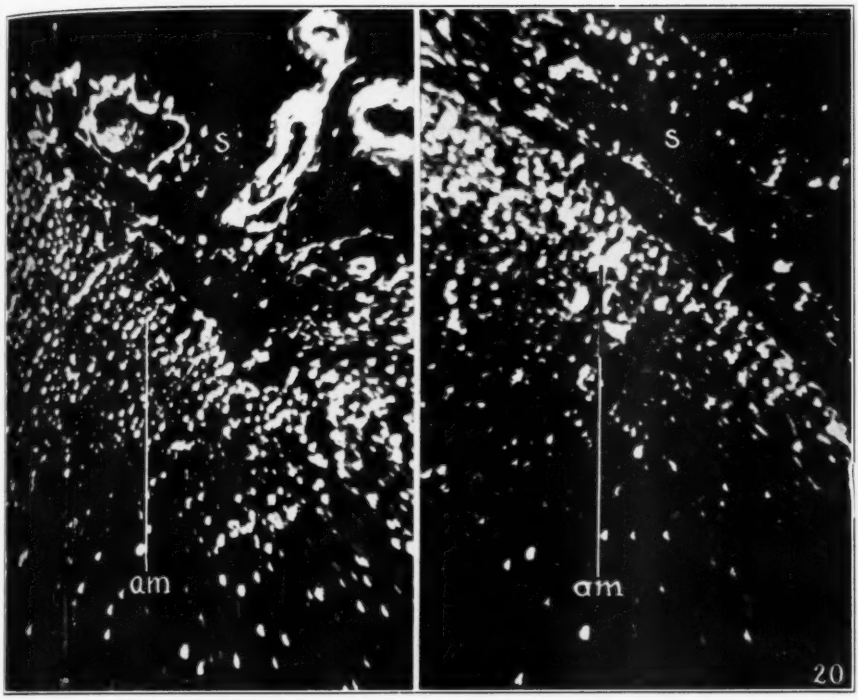

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PLATE 70

FIG. 23. Granular ependymitis in hemorrhagic superior polioencephalitis (Wernicke's disease) from the wall of the third (III) ventricle in a case of incipient delirium tremens, chronic alcoholism and avitaminosis, complicated by cardiac decompensation and syphilis. Female, 40 years of age. e = ependyma; s = subependymal tissue. × 110.

FIG. 24. Diseased extracerebral (meningeal) artery in cerebral arteriosclerosis from a 56 year old male. Branch of the arteria chorioidea. lb = lumen of blood vessel; ip = intimal proliferation; iem = inner elastic membrane; iemd = inner elastic membrane, delaminated; iemi = inner elastic membrane, inner lamina; m = media. × 115.

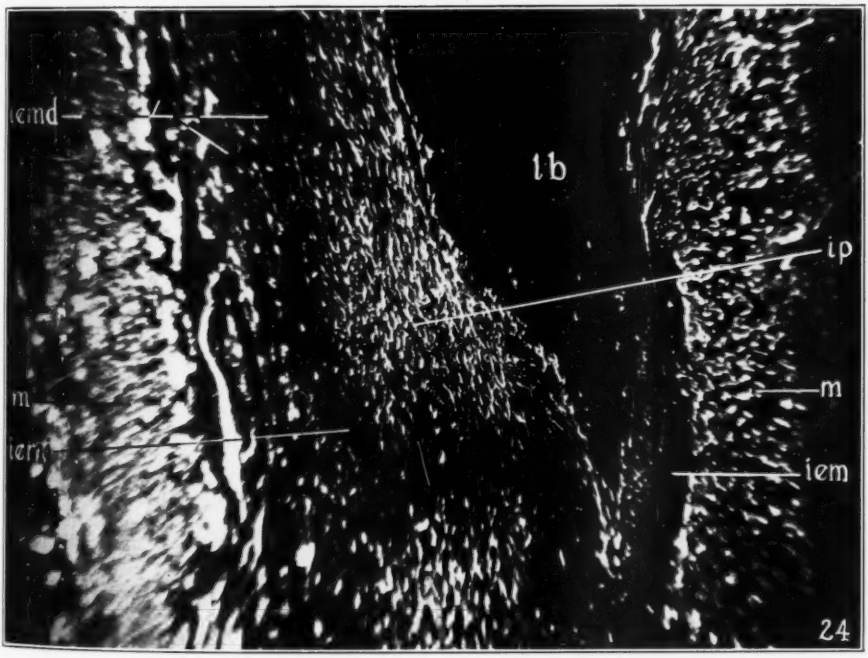

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PLATE 71

FIG. 25. Perivascular hemorrhage. From the periventricular region of the hypothalamus in a case of superior hemorrhagic polioencephalitis in a 40 year old female. b = lumen of blood vessel; ph = perivascular hemorrhage. (From the same case illustrated in Fig. 23.) × 500.

FIG. 26. Focus of softening in the central white matter of the brain. Cerebral arteriosclerosis in a 56 year old male. s = focus of softening; n = normal brain tissue. × 100.

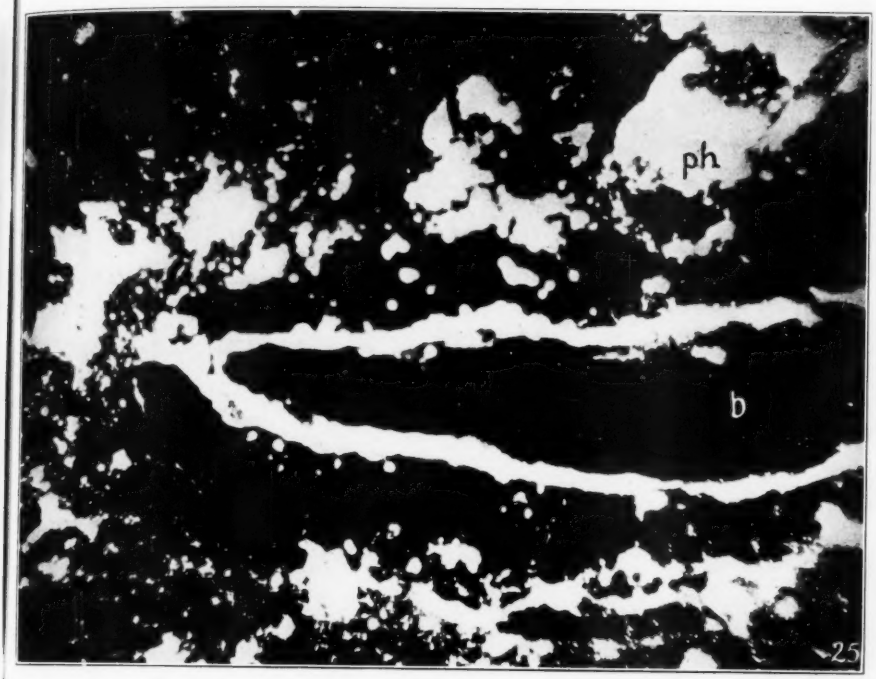

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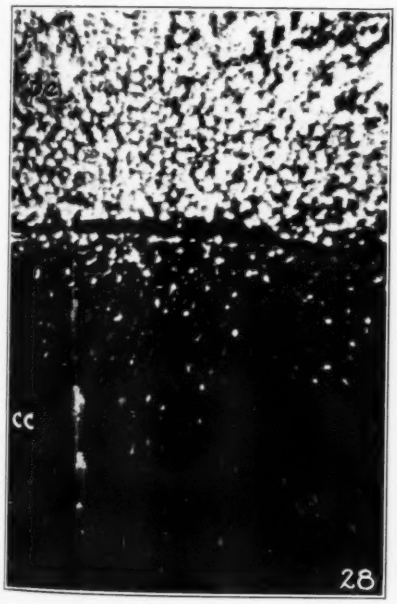
PLATE 72

FIG. 27. Thrombosed extracerebral (meningeal) artery. Cerebral arteriosclerosis in a 56 year old male. × 115.

FIG. 28. The purulent meningeal exudate (pe) covering the cerebral cortex (cc) in a case of pneumococcus meningitis. Male 49 years old. × 115.

FIG. 29. Perivascular polymorphonuclear infiltration (pi) of an intracortical cerebral blood vessel in an area of purulent meningoencephalitis in a case of pneumococcus meningitis. Male 49 years old. × 115.



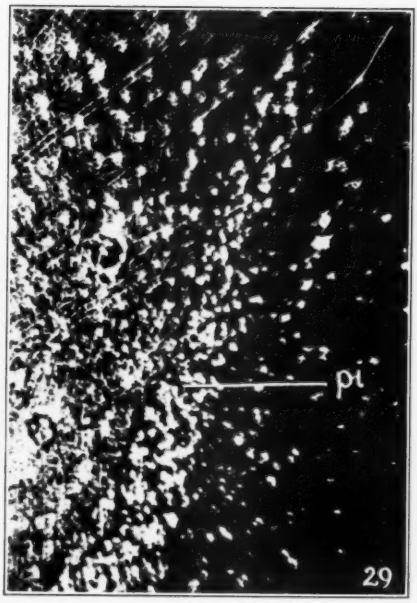


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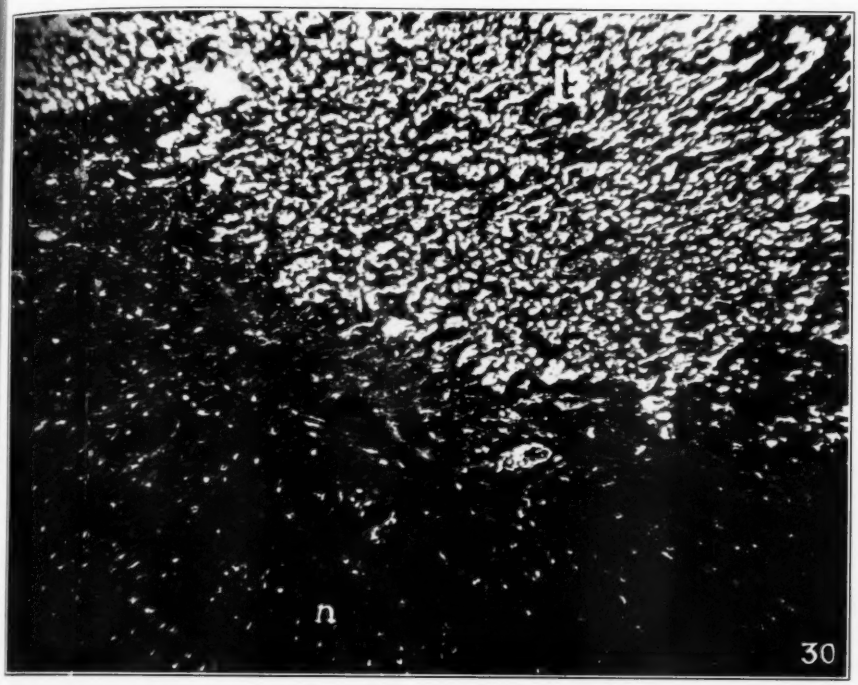
PLATE 73

FIG. 30. A metastatic tumor nodule in the brain (primary carcinoma of the bronchial epithelium with squamous metaplasia) from a 73 year old male. t = tumor; n = normal brain tissue. × 115.

FIG. 31. Tumor cells seen in Figure 30 (cerebral metastasis from primary carcinoma of the bronchial epithelium with squamous metaplasia) at higher power. × 540.



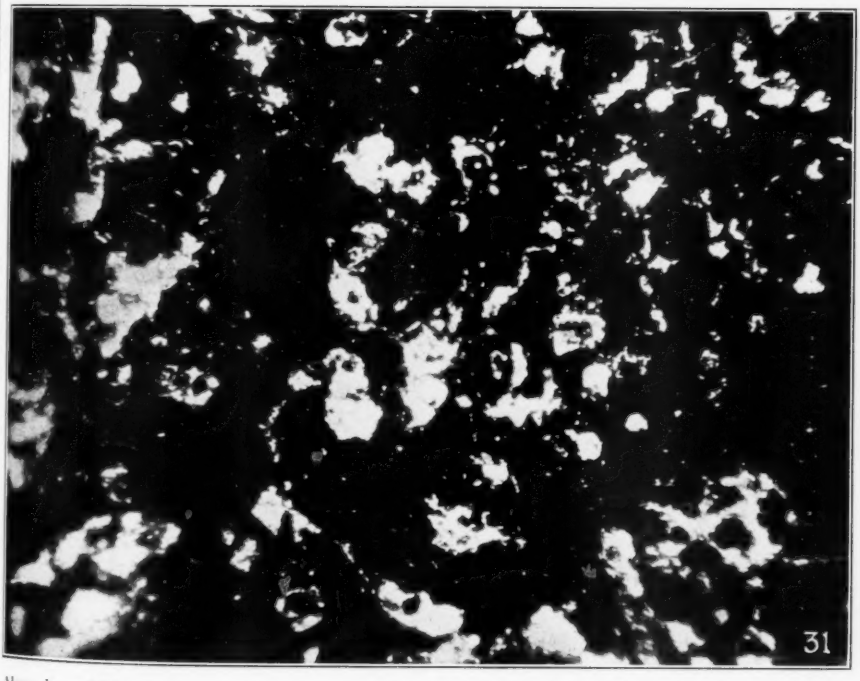


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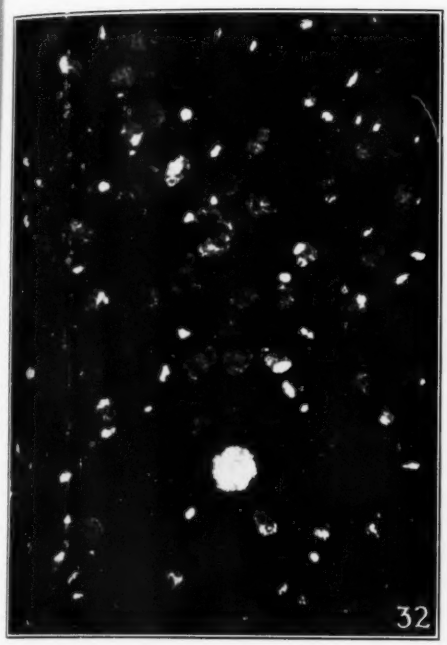
FIG. 32. Normal blood smear, human adult. × 530.

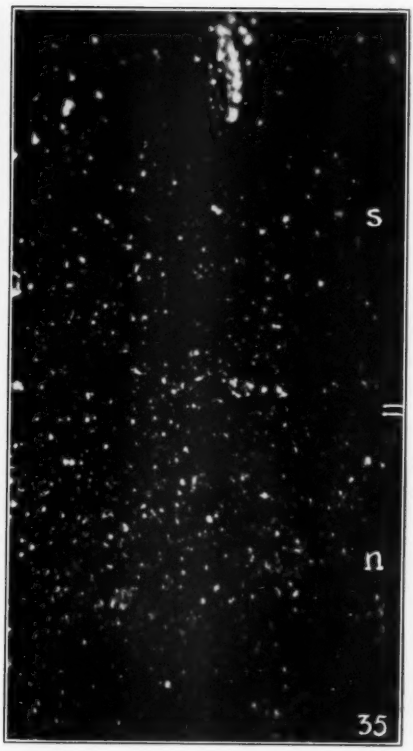
FIG. 33. A gumma (G) in the right substantia perforata anterior in a case of dementia paralytica. Male 45 years old. Oblique transillumination. × 8.

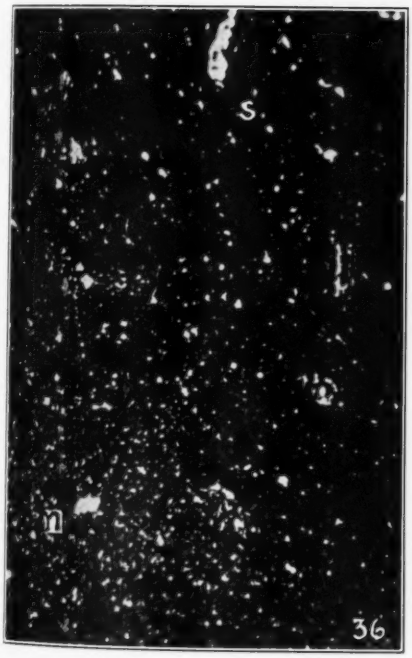
FIG. 34. A sclerotic plaque in the subcortical white matter of the brain from a case of multiple sclerosis. s = sclerotic plaque; n = normal white matter; g = normal gray matter. Microincinerated preparation (frozen section) 10 μ in thickness. Oblique transillumination. × 7.

FIG. 35. From a sclerotic plaque in the subcortical white matter in a case of multiple sclerosis in a 44 year old female. s = sclerotic plaque; n = normal brain tissue. × 110.

FIG. 36. From a sclerotic plaque in the subcortical white matter. Multiple sclerosis in a 44 year old female. s = sclerotic plaque; n = normal brain tissue. × 110.







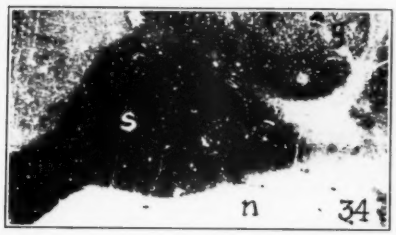


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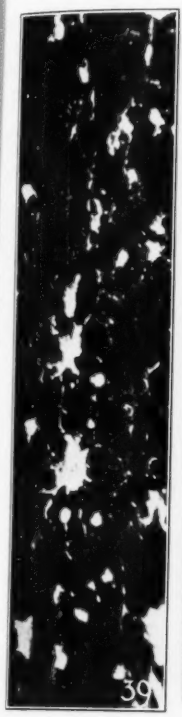
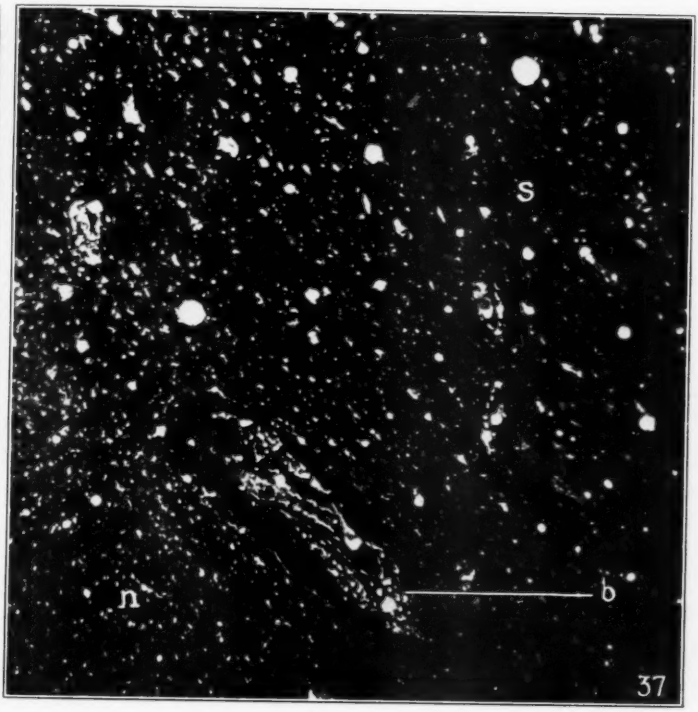
PLATE 75

FIG. 37. An early, hyperemic plaque in the subcortical white matter of the brain from a case of multiple sclerosis in a 44 year old female. s = sclerotic plaque; n = normal tissue; b = blood vessel on edge of plaque with perivascular infiltration consisting of lymphocytes and large phagocytic elements loaded with yellowish red mineral granules. × 110.

FIG. 38. A large phagocytic histiocyte containing granules of iron oxide in the infiltrated adventitia of a blood vessel from an early subcortical plaque from a case of multiple sclerosis in a 44 year old female. × 520.

FIGS. 39 and 40. Protoplasmic glial astrocytes in an early plaque in the subcortical white matter from a case of multiple sclerosis in a 44 year old female. In the center of Figure 40 two cross sections through a congested small vein are seen, with perivascular accumulation of lymphocytes and phagocytic histiocytes. × 520.





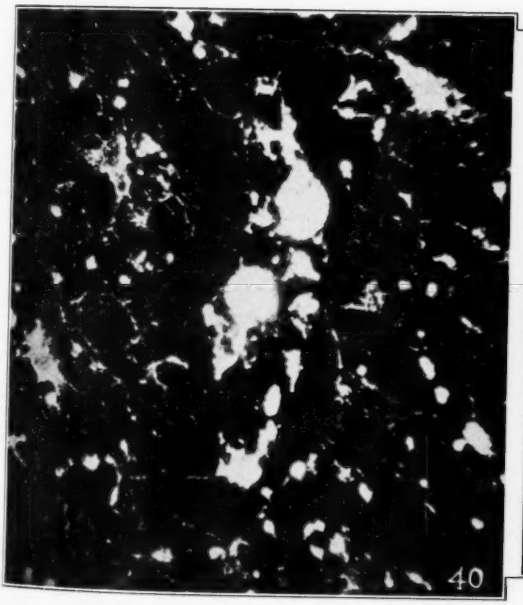


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FIG. 41. An old anemic plaque in the subcortical white matter of the brain from a case of multiple sclerosis in a 50 year old female. Note the nuclei and processes of fibrillary astrocytes visible at low power. × 110.

FIG. 42. Fibrillary glial astrocyte in an old plaque in the subcortical white matter at higher power. From a case of multiple sclerosis in a 50 year old female. × 520.

FIG. 43. The cornu ammonis (field H 4 and the fascia dentata) in a case of Alzheimer's disease (focal senile atrophy) in a 74 year old female. Note three argentophilic senile plaques (S). Paraffin section stained by a modification of Bielschowsky's silver impregnation. Light field transillumination. × 97.

FIG. 44. Neighboring section of Figure 43. Note the absence of appreciable traces in the mineral picture at the sites corresponding to the senile plaques in Figure 43. × 97.

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Fig. 45. One of the senile plaques seen in Figure 43, dorsal to the fascia dentata, to the left of a precapillary blood vessel, (b) at higher power. s = senile plaque. From a case of Alzheimer's disease in a 74 year old female. Modification of Bielschowsky's silver impregnation. Light field transillumination. × 514.

Fig. 46. Microscopic field corresponding to Figure 45 in a microincinerated neighboring section. The site corresponding to the senile plaque seen in Figure 45, dorsal to the fascia dentata, to the left of the same precapillary blood vessel (b) in this microincinerated preparation at higher power is marked only by a slight disarrangement of the glial reticulum, however, not by demineralization or by excess mineral residue. × 514.

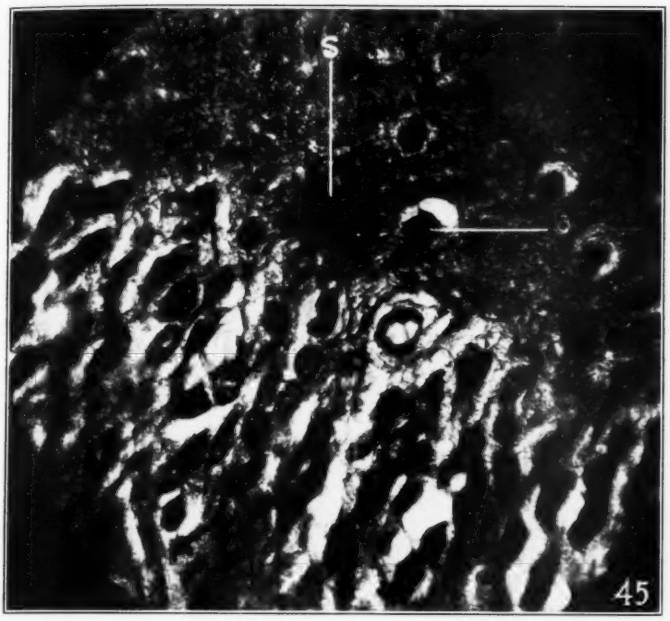

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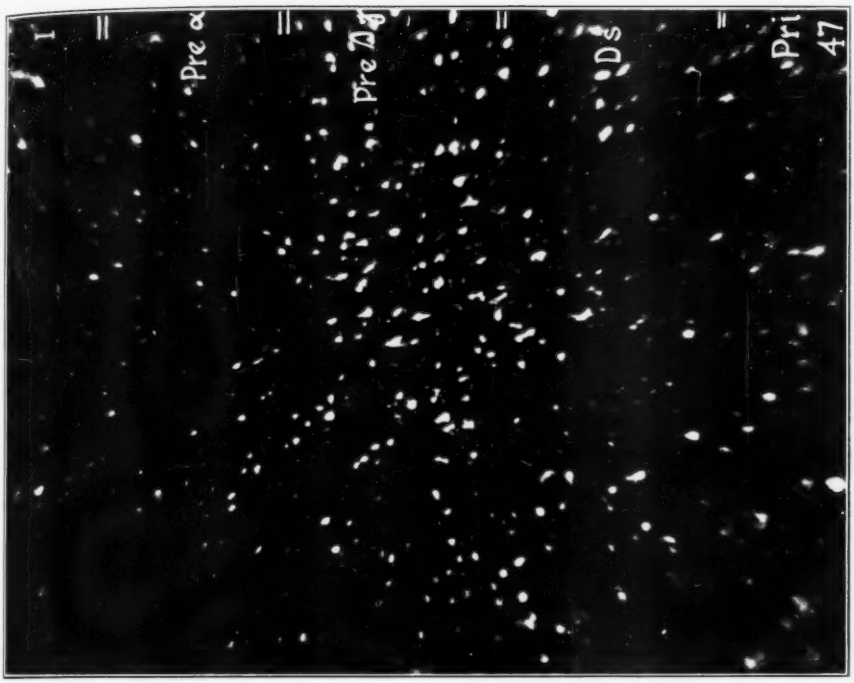
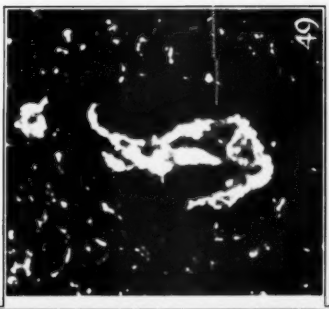
FIG. 47. The area entorhinalis in Pick's disease (focal senile atrophy), in a 72 year old female. 1 = lamina zonalis; Pre-*Alpha* = lamina principalis externa, sublayer *Alpha*; Pre-*Beta* and *Gamma* = lamina principalis externa, sublayers *Beta* and *Gamma*; Ds = lamina dissecans; Pri = lamina principalis interna. × 110.

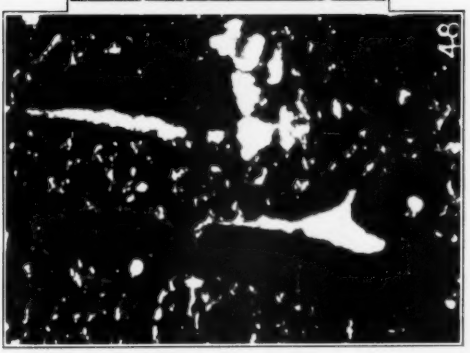
FIG. 48. A pyknotic (shrunken) ganglion cell and a glial rosette from the cortex of the subiculum cornu ammonis in a case of Alzheimer's focal senile dementia in a 74 year old female. × 1000.

FIG. 49. Alzheimer's ganglion cell disease in a ganglion cell of pyramidal type from the cortex of the subiculum cornu ammonis. Alzheimer's disease (focal senile dementia) in a 74 year old female. The characteristic thickened, winding neurofibrillar strands stand out by their substantial mineral content, while the remainder of the cytoplasm of the cell is demineralized. × 1500.

FIG. 50. Three ganglion cells from the cortex of the subiculum cornu ammonis in a case of Alzheimer's disease in a 74 year old female. × 1000.







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STUDIES ON THE EXPERIMENTAL INFECTION OF SOME REPTILES, AMPHIBIA AND FISH WITH *SERRATIA ANOLIUM* *

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In a preceding paper, Duran-Reynals and Clausen [1] (1936), a microorganism was described as the causative agent of a tumor-like disease found in a species of Cuban lizard, *Anolis equestris*. The organism is a motile, Gram-negative rod, producing a pink pigment under certain conditions. It has been temporarily classified as belonging to the family *Bacteriaceae*, tribe *Chromobacterieae*, genus *Serratia*. This organism was described in a previous paper as *Serratia anolium*.

In this investigation we have studied the pathogenic effects of *S. anolium* on many cold-blooded animals, including the species in which the disease was originally discovered. A point of general interest, namely the action of temperature on infection, which affects many species of cold-blooded animals, was especially fitted for such studies.

MATERIAL AND METHODS

The animals used in the following experiments were as follows: The iguanid lizards, *Anolis equestris* and *Anolis carolinensis*; the gekkonid lizards, *Tarentola mauritanica* and *Hemidactylus brookii*; the snakes, *Thamnophis butleri* and *Storeria dekayi*; the musk turtle, *Sternothaerus odoratus*; the toad, *Bufo americanus*; the frog, *Rana pipiens*; and the catfish, *Ameiurus melas*.

All of the above animals used for inoculation tests were selected from the stock animals which had been kept in the laboratory for some time and hence had become adjusted to laboratory conditions. Healthy specimens of *A. equestris* were also secured from Cuba during the course of the experiment.† They were kept under observation

* Received for publication November 13, 1936.

† We are greatly indebted to Dr. F. Ramirez-Corria of the Findlay Institute of Havana for sending these lizards.

for several days before inoculation. The experimental animals were placed in individual cages or aquaria and food and water were kept in the containers at all times. The food consisted of a supply of larvae of the meal beetle (*Tenebrio molitor*) and the wax moth (*Galleria mellonella*). Earthworms were also placed in the cages or aquaria containing the snakes or fish.

Twenty-four hour cultures in broth or agar, suspended in 10 cc. of saline, were used for the inoculations. In a few cases the animals were directly grafted with tumor-like masses.

Further details regarding specific methods will be included in the respective sections of the results to follow.

Experimental

Inoculation of Anolis equestris

In different experiments lasting several months 6 *A. equestris* were injected subcutaneously in the hind limb with a 0.2 cc. suspension of agar culture. Swelling developed in all animals within 2 to 3 days after the injection. This swelling increased considerably during the following week. In some cases the infection localized in the knee joint and resulted in the formation of a voluminous round mass (Fig. 1). This mass persisted for several months and gradually disappeared. In 3 other cases the swelling was more diffuse (Fig. 2) and the animals died within 6 to 7 weeks after the injection. Postmortem examination of 2 of the lizards showed degeneration of the tissues which exhibited a yellowish color and were surrounded by an area of hemorrhage. The causative organism was readily recovered from the injected site, and from the heart blood as well. In the 3rd lizard postmortem examination showed that the muscle of the injected limb was "shot" with small nodules. Large nodules with necrotic centers were found in the deeper layers. A small mass of hard opalescent tissue with a necrotic center was also found beneath the angle of the jaw. The liver was also "peppered" with pinhead whitish areas. The organism was recovered from the hind limb, the nodule in the jaw region, the liver and also from the peritoneal cavity. In another *A. equestris*, similarly injected, a chronic process developed with the formation of a nodule persisting for several weeks. Finally, in another test 1 animal was injected in the splanchnic cavity without any resulting ill effect. Of the above mentioned

animals, 4 were additionally inoculated by scarification of toes and vocal pouch. No infection resulted after such treatment.

It may be concluded from these tests that cultures of *S. anolium* injected into *A. equestris* result in the production of a condition which, in its chronicity and localized character, resembles the natural disease of the same animal.

Effect of Environmental Temperature on the Experimental Disease in Anolis equestris

Dealing with an infection of cold-blooded animals it was obviously interesting to study the modification of the infection when the temperature was elevated. Accordingly, some tests were carried out on *A. equestris* in which the action of *S. anolium* was studied with higher temperatures.

In one experiment 2 lizards were injected subcutaneously with 0.5 cc. of saline suspension of a 24 hour agar culture. Immediately afterwards 1 of the animals was placed in the incubator at 37° C. Swelling developed in both animals during the first several days, but at the end of the 3rd day the swelling had practically disappeared in the animal kept at 37° C., whereas it steadily increased in the control animal left at room temperature. The animal at 37° C. lost considerable weight and died on the 5th day. By that time the animal kept at room temperature showed a marked inflammation of the entire thigh. It was then placed in the 37° C. compartment. In the course of the next 2 days the inflammation subsided and was limited to a small region in the anterior portion of the thigh. The animal appeared sick, lost weight and died on the 7th day after being placed at 37° C. The bacterium was abundantly found in the injected thigh and the blood.

In a second experiment 2 more *Anolis* were injected as described above. Ten days later both animals showed a slight swelling in the injected region. One of them was placed at 37° C. and the other was left as a control at room temperature. In the former lizard the inflammation subsided; the animal was taken sick and died 7 days after being placed at 37° C. The causative bacteria were also isolated from the injected region and from the heart. The control animal at room temperature developed a lesion in the knee which lasted several months and finally disappeared, leaving a permanent deformity of the bones.

As a further control to these experiments a non-injected *A. equestris* was left at 37° C. for several days. No alteration in its health was apparent.

Effect of Staphylococcus aureus, Streptococcus hemolyticus and Meningococcus on Anolis equestris

It was desirable to know whether the experimental lesions obtained in *A. equestris* by *S. anolium* were of a specific type or could be reproduced by agents pathogenic for mammals, such as staphylococcus, streptococcus and meningococcus. Accordingly, the *A. equestris* were each injected under the skin of the thigh with 0.2 cc. of saline suspension of a 24 hour agar or blood agar culture of the above mentioned bacteria. In the case of meningococcus, mucin was added to the inoculum in order to enhance the infective power of the bacteria. After injections were made the corresponding bacteria were seeded in adequate media and left at room temperature, as were the injected lizards. In each case a growth was obtained. The results of these tests may be summarized as follows. A localized inflammation without any specific character resulted immediately after the injection. This remained stationary for a few days and had practically disappeared after 2 weeks. No general ill effect could be noticed.

In another test 1 lizard was injected with meningococcus mixed with mucin and left at 37° C. for 2 weeks. The result was identical with that shown when the animal was kept at room temperature.

Thus, 3 bacterial species of marked pathogenic power for mammals proved to be devoid of any serious infective power for *A. equestris*.

Anolis carolinensis

After having reproduced the disease in the species which originally showed it, it was thought desirable to study the susceptibility of other cold-blooded animals. A species of lizard related to *A. equestris*, namely *A. carolinensis*, was first used.

In the first experiment 5 *A. carolinensis* were directly grafted under the skin of the thigh or the foreleg with small portions of the healthy viscid tissue of a nodule recently removed from an *A. equestris*. All the animals developed an acute condition in the injected region and 2 of them died 10 and 23 days, respectively, after the in-

jection. Locally, an abscess was formed from which the organism was recovered. It was also recovered from the blood. In the other lizards a localized and raised tumor-like condition developed. This condition either persisted for several weeks without rupturing or a suppurative process was established which lasted for an extended period. Lesions healed after 2 to 3 months, leaving a hard nodule.

TABLE I

Results of Various Concentrations of Inoculum from 24 Hour Cultures of Serratia anolium on the Lizard, Anolis carolinensis

No. of cases	Dilutions *	Lesions	Cultures †
	cc.		
2	0.02	Positive	From nodules & heart
2	0.005	Positive	From nodules
2	0.002	Negative	From heart in 1 case
3	0.001	Negative	From heart in 1 case
2	0.0001	Negative	Negative

* Dilutions were made from 24 hour cultures in broth or agar, suspended in 10 cc. of saline.
† Animals were sacrificed 3 months after injection and cultures and smears were made at that time.

In another test 2 *A. carolinensis*, which were left from the initial experiment and which still exhibited nodular lesions in the foreleg, were injected in the opposite foreleg with 0.1 cc. of a 24 hour broth culture of the bacterium. As a control, 2 normal lizards of the same species were similarly injected. All the lizards died between the 3rd and 6th day after inoculation. The causative bacterium was recovered from the local lesion and from the blood of the entire group of animals.

In a third series of experiments it was thought of interest to determine, if possible, the concentration of the inoculated medium necessary to produce the typical lesions. Hence, a series of concentrations was made. Dilutions were all made from 24 hour cultures suspended in 10 cc. of saline. Each animal was injected in the thigh of the hind limb with 0.1 cc. of the suspension. The injected lizards were kept in one cage at 24° C. with 5 normal non-injected animals.

The results, as recorded in Table I, show that, up to a certain point, the localization and nodule formation were dependent on the dilution and that as the concentration decreased beyond this point the lesions failed to appear. Nevertheless, the latter low concentrations often remained virulent and were found in the heart blood

for a considerable period even though no local lesions occurred. The normal lizards, on postmortem examination, revealed no evidence of the causative bacterial organism.

Inoculation of Gekkonid Lizards

Since experiments on *A. carolinensis* had shown that the bacterial organism *Serratia anolium* would produce pathogenic effects comparable to those produced in another species of lizard of the same genus, it was of importance to repeat the inoculations in an entirely different group of lizards.

Three *Tarentola mauritanica* lizards were injected subcutaneously in the left hind limb with 0.2 cc. of the suspension from a 24 hour culture. Two of the lizards were kept at room temperature and the other was placed at 37° C. The 2 former showed a slight swelling in the injected area 10 days after the injections and lesions developed in the injected limbs. These lesions continued to enlarge during the month following (Fig. 3) and the bacterium was recovered from the lesions and also from the heart blood 2 months following injection. The increase in size of the limbs had apparently subsided at this time but no decrease in size could be noted. The lizard at 37° C. died 11 days after the injection and the causative organism was isolated from the injected region, the peritoneal cavity and the heart blood. These experiments were then repeated on another gekkonid lizard, *Hemidactylus brookii*, and the results were similar to those obtained in *T. mauritanica*.

Experiments with Turtles

Since the bacterium *S. anolium* produced tumor-like lesions in several species of lizards, it was thought necessary to repeat some of the above tests on other reptilian forms. Hence, the musk turtles (*Sternothaerus odoratus*) were selected. Two turtles were injected with 0.2 cc. of the inoculum into the right hind limb and into the foot of the left forelimb, respectively. No swelling or nodule formation was noticeable for 2 months in either of the turtles. After 3 months the region of injection showed rapid signs of enlargement. These nodules increased in size very rapidly during the following month (Fig. 4), and at the end of the 4th month both turtles were sacrificed. The causative bacterium was easily isolated from the lesions as well as from the heart blood in all instances.

Experiments with Snakes

The garter snakes, *Thamnophis butleri*, and the brown snakes, *Storeria dekayi*, were selected for injection to see if the bacterium, *S. anolium*, would also produce tumor-like lesions in this group of reptiles. Accordingly, 3 *T. butleri* were injected, subcutaneously, about 3 cm. caudal to the head region. Two snakes were left at 24° C., while the 3rd was placed at 37° C.

In the case of the 2 snakes at 24° C., the nodules began to form 3 weeks after injection and reached their maximum size at 6 weeks (Fig. 5). After 6 weeks the nodules failed to increase in size but continued to subsist for a period of 4 months. The bacterium *S. anolium* was recovered from the lesion and from the heart blood in both cases. In the snake at 37° C. slight swelling was evident 3 days following injection but the animal died 9 days after injection. The causative bacterium was recovered from both the lesion and the heart blood.

These experiments were then repeated on another species of snake (*Storeria dekayi*) and exactly the same results were obtained.

Experiments on Amphibians

Two frogs, *Rana pipiens*, and 2 toads, *Bufo americanus*, were injected with 0.2 cc. into the hind limb. At the end of 4 days a noticeable swelling was evident in both species and at the end of 3 weeks the injected portion of the thigh had increased three times its normal size. In 1 of the toads the integument over the enlarged nodule was broken. In this latter case a mixed culture was obtained but the bacterium *S. anolium* was included. In the case of the 2 frogs and the remaining toad the animals were sacrificed 1 month following injection. Cultures of the bacterium were obtained from the lesions in all 3 instances. The heart blood also contained the causative organisms in all 3 cases. In 1 frog the tibia bone was broken (Fig. 6) and microscopic sections showed a degeneration of the tissue with the bacterium invading the marrow of the bone as well as the surrounding tissues.

Injection Experiments on Fish

Three small catfish, *Ameiurus melas*, were selected from a group of 6 which had been brought into the laboratory 2 years before, and

injected with 0.1 cc. of the inoculum. Injections were made on the left side approximately equidistant from the head and caudal fin. The 3 injected fish were placed in the same aquarium with 3 non-injected catfish. At the end of 3 days the area of injection became quite necrotic and the integument and underlying tissues sloughed (Fig. 6). Ten days later this area had become completely healed and the fish showed no lesions and appeared normal in all respects. At the end of 3 months all the fish were sacrificed and the bacterium was recovered from the heart blood of not only the injected ones but 2 of the 3 non-injected ones as well.

These results show that the bacterial organism in question is retained in the fish for at least 3 months and, when cultured, shows all of the growth characteristics of the bacterium from other cold-blooded forms tested even though lesions were never produced in this species of fish. It was also of interest to show that the organism could be passed through the medium of the water to a non-injected specimen.

Tests with the Rabbit, Guinea Pig and Mouse

After having studied the effects of *S. anolium* on cold-blooded animals it was obviously interesting to observe the effects it might have on some of the mammals commonly used in laboratory research.

Two rabbits were injected intracutaneously with 0.5 cc. of a 24 hour culture and a 3rd rabbit with 0.2 cc. of the same culture intracutaneously. The 2 former rabbits developed a necrotic lesion 3.5 by 5.5 cm., which was well localized and healed in 10 to 15 days. The latter animal did not show any symptoms of the disease whatsoever.

One guinea pig was injected in the skin and another in the peritoneum with 0.2 cc. of the same culture. In the former animal a round, slightly necrotic lesion, about 4 cm. in diameter, developed. This healed in approximately 10 days. The latter animal did not show any appreciable sign of the disease.

Ten mice were injected with doses ranging from 0.1 to 0.0001 cc. in the leg muscle. A slight swelling and redness developed in only 1 animal, the animal injected with the largest quantity. This reaction, however, disappeared after 5 days. In another experiment 2 mice were injected intracutaneously with 0.1 and 0.2 cc., respectively, of a 24 hour broth culture. The mouse injected with 0.2

cc. died with an acute inflammation and necrosis of the abdominal tissues. The other mouse showed no apparent sign of the disease.

It can be concluded from these experiments that, as contrasted with the relatively high pathogenicity produced by *S. anolium* in amphibians, reptiles and to some extent fish, it is little or non-virulent for the mammals so far tested.

Discussion

Experimental tests have designated that the increase of temperature of a cold-blooded animal to an approximate level usually shown by mammals brings about a considerable modification in respect to this bacterial infection. What, under normal surrounding temperature (18°–24° C.) is generally a chronic, well localized process, which rarely generalizes, becomes a general disease which results in the death of the animal in a few days if the temperature is increased to 37° C. At room temperature death was never observed prior to 6 weeks after injection. Co-existing with the generalization there is a considerable improvement and often times complete disappearance of the local lesion. It is possible that the local gain might be obtained at the expense of the invasion of the animal tissues by the bacterium.

On one hand, this series of experiments offers an analogy with, and might be considered the counterpart of, the classic experiment by Pasteur, who succeeded in infecting chickens, a naturally refractory bird, with anthrax by lowering the temperature of the bird after the experimental inoculation with *B. anthracis*.

Considered from another angle, the described results may be compared with experiments reported by Wollman and Uribe [2] (1931). These investigators observed that no antibodies against red cells and bacteria could be produced when these antigens were injected into a frog, *Calyptocephalus gayi*, kept at normal temperature. However, they obtained rather high titers when the temperature of the animals was increased. Other investigators, Dieudonné [3] (1894), Schire and Greenfield [4] (1929), and others, have also shown the differential bacterial reactions in cold-blooded forms when temperatures were altered and have also compared their effects on various warm-blooded animals.

During the course of this series of experiments a number of iguanid lizards, *Basiliscus vittatus*, were sent to the Museum labora-

tory from southern Mexico. Upon their arrival, a tumor-like lesion was noted on the hind limb of one of the animals. Cultures were made and the causative organism isolated. This bacterium was found to be the same species (*Serratia anolium*) as found in *Anolis equestris*. These findings are of special interest in that they serve to illustrate the more extensive distribution of this organism in nature.

The results as presented in this series of experiments should serve as additional stimulation toward speculation concerning infection and resistance in higher animals, and also to emphasize the importance which comparative immunology would have for a better understanding of these processes.

Summary

1. *Serratia anolium*, a bacterium which induces tumor-like lesions in the iguanid lizard *Anolis equestris*, was injected into other cold-blooded animals as well as several species of warm-blooded forms.

2. In all the forms of reptiles or amphibians used the lesions were produced and appeared to be similar in all respects to the natural disease. The causative bacterium was easily isolated and cultured from the lesion as well as from the heart blood.

3. In the experimentally injected cold-blooded forms which were kept at room temperatures, the bacterium, while being found in the blood, usually became localized and produced lesions. Diversely, the higher temperature (37° C.) for the same species of experimental animals, always produced general pathogenic effects and proved fatal in most instances.

4. In the warm-blooded animals used, the bacterium was non-virulent and no pathogenic effects were noted in any case.

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DESCRIPTION OF PLATE

PLATE 79

FIG. 1. *Anolis equestris* injected with *Serratia anolium* in left thigh. Infection localized in the knee joint. Note voluminous round mass.

FIG. 2. *A. equestris* injected with the bacterium in left thigh. Swelling most diffuse but localized. This size remained as shown for 7 weeks until the animal died.

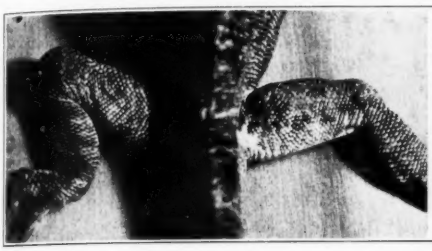
FIG. 3. *Tarentola mauritanica* injected subcutaneously in left hind limb 4 weeks after inoculation.

FIG. 4. Ventral view of musk turtle showing nodule formation in right hind limb 4 months after inoculation.

FIG. 5. The garter snake, *Thamnophis butleri*, showing nodule formation on the right side 6 weeks after inoculation.

FIG. 6. Injected hind limb of leopard frog dissected to show the broken tibia extending from the center of the mass to the extreme left.

FIG. 7. Inoculated catfish showing necrotic area in mid anterior-posterior region 3 days after injection of bacterium.



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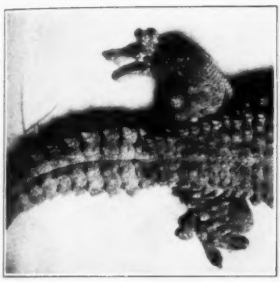

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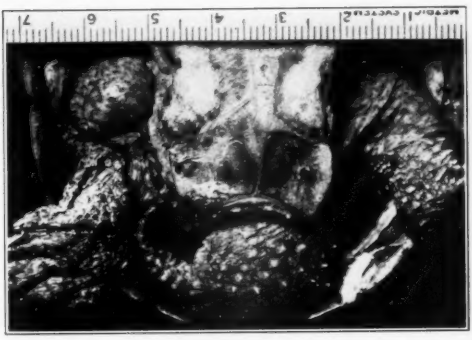

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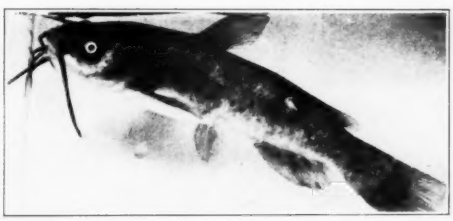

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6

Clausen and Duran-Reynals

Experimental Infection with *Serratia Anolium*

UNIV. OF MICH.

STRUCTURE OF THE SMALL CEREBRAL ARTERIES AND THEIR CHANGES WITH AGE *

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Many histologists believe that the arteries of the brain are anatomically different from those in other parts of the body; others feel that they are essentially the same. In reviewing the literature on the structure of normal cerebral vessels one is impressed by the paucity of reports. It was for this reason that our attention was directed to the investigation of the histology of the small cerebral vessels and the changes that occur in their walls with advancing age.

Triepel [1] in 1897 first described in detail the histological structure of the cerebral vessels. He suggested that they differed from similar sized vessels elsewhere in the body in three features: in the great prominence of the internal elastic lamina; in the slight development of the elastic tissue in the circular musculature of the media; and in the striking decrease in the longitudinal elastic fibers of the adventitia. He believed that as the artery decreased in size the elastica interna decreased in thickness until it finally disappeared entirely in the precapillary stage, leaving a vessel composed simply of a few strands of muscle fibers lined by a layer of endothelial cells.

Schäfer's [2] description in 1912 agreed with that of Triepel.

Binswanger and Schaxel [3] in 1917 studied the cerebral arteries in brains of individuals from birth to 60 years of age and concluded that at birth the structure of all arteries is complete and the later changes are those of quantity alone. As age advances the elements vary in their growth. By middle life the elastic tissue of the media and the elastica interna has stopped increasing. The muscle of the media continues to grow up to the 60th year. The collagenous tissue continues to increase and retains the greatest growing power. In older people, therefore, the cerebral vessels would show considerable increase in connective tissue at the expense of the rest of the wall elements. As the vessels decrease in size there is a decrease in the

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quantity and quality of the elements in the walls. The elastic fibers in the media disappear first, then the muscle fibers decrease, only the collagenous tissue remaining to surround the arteriole to the precapillary stage. This is in disagreement with Triepel who believed that muscle fibers could be detected around precapillaries.

Spielmeyer [4] in 1922 and Jacob [5] in 1927 agreed in their description of the cerebral vessels with Binswanger and Schaxel. However, they differed in their description of the capillaries which they believed contained an intima, elastica interna and a few adventitial fibers. Jacob described the cerebral arterioles as containing definite oblique muscle cells within their media.

Hackel [6] in 1928 studied the cerebral arteries in 13 cases varying in age from 15 days to 50 years, and described a structure quite similar to that described by Triepel. Hackel emphasized the relative scarcity of elastic fibers in the media as compared to the adventitia.

In 1931 Tuthill [7] made a study of the elastic layer of the cerebral arteries in 2 stillborn infants and in 24 children varying in age from birth to 14 years. He believed the size of the vessel determined the presence and the thickness of the elastica interna at birth. In the smaller arteries the elastic layer increases in thickness chiefly during the first 5 years, although it continues to grow throughout childhood, becoming quite a prominent part of the vessel wall.

With these earlier investigations in mind the present studies were undertaken in order to determine the normal histology of the cerebral vessels, as well as the normal variations in structure that may be encountered in the routine observation of normal brains in any single age group. For this investigation 70 apparently normal brains were selected, from our coroner's service, from individuals all of whom died of some extracerebral condition such as gunshot wound of the abdomen, crushed chest, and so on. The age groups of these individuals were as follows: 6 cases up to 10 years of age; 21 cases from 11 to 30 years of age; 20 cases from 31 to 50 years of age; 16 cases from 51 to 60 years of age; and 7 cases over 60 years of age.

During the early part of this investigation many blocks of tissue were taken from the various regions of the brain. It was necessary, therefore, to study a great many slides in each case. It was soon observed that the structure of the small cerebral arteries was quite constant in the various blocks studied, and that adequate observations could be made on fewer sections. Therefore, single blocks were

taken from representative areas throughout the cerebral cortex and the white substance and fixed in formalin. Sections were stained with the Weigert-Van Gieson stain, by the Mallory-Heidenhain technique (azocarmine), with Weigert's elastic tissue stain, and with the hematoxylin-eosin stain. For the study of muscle and fibrous tissue in the cerebral vessels staining by the Mallory-Heidenhain technique is invaluable since it demonstrates even the smallest connective tissue and muscle fibers. However, it does not differentiate between elastic and collagenous fibers. For this purpose Weigert's elastic tissue stain serves very well. Hematoxylin-eosin and Van Gieson stains were used to study the cellular structures.

The present study was limited to arteries varying in size from 20 to 150 μ in diameter. A detailed description of the larger vessels will be given, followed by a discussion of the various modifications that occur in the wall elements as the vessel decreases in size.

Normal Cerebral Arteries

The small cerebral arteries are characterized by the thinness of their walls. In arteries measuring 150 μ in diameter the vessel wall is frequently less than 15 μ in thickness. The walls of these vessels may be divided roughly into the three coats ordinarily described for arteries, namely, interna, media and externa, although microscopic study of these vessels does not always reveal clear-cut divisions between these layers. The middle layer shows the greatest departure from the structure usually observed in the average small artery. It will, therefore, be described in some detail in a later paragraph.

Intima: The inner coat or intima is composed of an endothelial lining and an elastica interna. The latter is a thick, compact laminated structure and exhibits only a few fairly regular waves that project into the vessel lumen. This internal elastic lamina is both relatively and absolutely thicker in the cerebral vessels than in similar vessels elsewhere in the body. This relative thickness of the elastica interna tends to persist even into the smallest arteries. In many of these smaller vessels, although this structure retains its relatively large proportions, it frequently reveals certain changes in its tinctorial properties. Irregular segments of the elastica may fail to stain. In the smallest of vessels the elastic membrane decreases rapidly in thickness until it finally consists of a few strands of fibers that appear as granules in cross section.

Media: The media of most small extracerebral arteries is usually thick relative to the size of its lumen and consists mostly of circularly arranged flat bundles of smooth muscle. Numerous elastic fibers appear between the muscle cells forming a wide meshed network. Delicate collagenous strands, although present within the media, are few in number. They tend to follow the ramifications of the elastic fibers into the adventitia. The basic tissue within the media of the average small artery of a young individual consists, therefore, of smooth muscle, and the collagenous fibers are usually few and inconspicuous. The latter tissue does increase with advancing age, but only in vessels of very old individuals does it become a dominant element in the media. The middle layer of the small cerebral arteries presents quite a different picture. It is composed of a foundation of radially arranged, fine and coarse collagenous fibers which seem to fuse with one another. The collagenous tissue comprises a surprisingly large proportion of this lamina and in many cases makes up the greatest bulk of the wall (Fig. 1). Throughout this connective tissue there are found varying amounts of obliquely arranged muscle cells and nuclei. The muscle tissue is usually quite irregular in occurrence and is often entirely absent from a part or from the entire vessel wall. The elastic elements in the media are scarce.

There is a striking change in the middle layer as the vessel decreases in size. The structure of the wall becomes more simple. The elastic and muscle tissue rapidly disappear and are often difficult to find in vessels of 70 μ in diameter. Often only an occasional muscle cell nucleus can be detected within an almost solid collagenous wall. Thus in the smaller vessels the entire media, regardless of the age of the patient, appears to be composed of collagenous connective tissue, which merges imperceptibly with the adventitia. At this stage, with the azocarmine stain, this lamina is a solid blue staining layer of tissue.

The media of the small cerebral arteries, therefore, possesses certain features that are quite different from those of similar sized vessels elsewhere in the body, namely, a relative paucity of both elastic and muscle tissue and a predominance of collagenous fibers. In all the smaller vessels, as well as in many of the larger ones of apparently normal young adults, the media is composed almost strictly of collagenous tissue. So striking is the appearance of this

layer, especially when stained by the Mallory-Heidenhain technique, that at first it was believed to be of pathological significance. Its uniform occurrence in the smaller arteries demonstrates that what appears to be fibrosis of the media is normal for cerebral arteries.

Adventitia: The adventitial layer of the small cerebral arteries is quite variable in size. In some cases it is composed only of a few strands of tissue, while in others it is equal in thickness to the adjoining media. As a rule it is quite thin and made up of a loose network of longitudinally and circularly arranged collagenous fibers. This lamina is well supplied with elastic fibrils. In the smaller arteries this outer layer of collagen becomes less and less distinct as an individual structure. It merges with the adjacent fibrous media to form a single layer.

Age Changes

Intima: With the advance of age numerous alterations begin to occur in all layers of the cerebral arteries. One of the first structures to show changes is the internal elastic lamina. Whereas this structure is usually a solid uniform band, it begins to show areas of reduplication as early as the latter part of the third decade. At first this consists of an irregular fraying of the solid, thick elastic lamina, forming tiny fine fibrils which project irregularly from various portions of the main lamina. This process results in a pseudoreduplication of the internal elastic lamina. True multiplication of the elastica interna does occur, but usually later in life — during the fourth decade and most frequently during the fifth and sixth decade (Fig. 2). This reduplication, however, is not the only change that occurs within this structure with the advance of age. Changes of equal frequency occur in the tinctorial properties of this membrane. It loses its ability to stain normally with the usual dyes and appears patchy and indistinct (Fig. 2).

Occasionally certain segments of the elastic lamina appear swollen. This swelling usually extends in an outward direction encroaching on the media. It is in these swollen areas that the first changes in the staining properties of this elastic tissue occur.

In advanced age large portions of the elastic membrane lose their normal staining properties. The thickened pale staining membranes, instead of appearing as uniform structures, are split not only into longitudinal structures but often also into transverse frag-

ments. This, no doubt, is a stage in the final disintegration of this membrane.

Media: The earliest change that is noticeable in the media of the cerebral arteries is an alteration quite similar to that which occurs as the vessel decreases in size, namely, a complete reduction in the quantity of its elastic and muscular elements. This change is quite conspicuous and is fairly complete early in life. When fibrosis of the media is complete it is usually impossible to differentiate this lamina from the adventitia, these two layer elements merging with one another to form a single structure. The cerebral vessels usually reveal a complete fibrosis of their media long before any preponderance of collagen begins to be apparent in this layer of the extracerebral vessels. Hence, such vessels in the younger age group must not be considered as a pathological finding but as a normal age process that occurs fairly early in life.

Other age changes occur within this layer. During the fifth and sixth decades many of the vessels show an indistinct staining of the medial elements. The heavy collagenous fibers lose their outline and become hyalinized. Similar changes occur in the adventitia which at this stage usually cannot be separated from the media (Fig. 3).

The extreme fibrosis and hyalinization of the vessel elements often fray the arterial wall and weaken it to such an extent that erythrocytes break through and escape from the lumen through the frayed elastica and vessel wall to the perivascular spaces, forming a ring of red cells around the vessel. It is not uncommon to find red cells scattered among the fragmented elements of a hyalinized vessel wall.

A final and more uncommon alteration that is observed in the media with advance of age is calcification of the wall elements. This change was rarely observed before the fifth decade. The calcium is at first deposited as irregular tiny particles in the outer portion of the media and inner adventitial layers (Fig. 4). The deposition may increase and become so complete that the entire wall is replaced by the calcium particles, which form a solid ring around the uninvolved lumen. This calcification is independent of any other change within the wall and may occur with or without hyalinization or fibrosis of the wall elements.

Adventitia: The age changes in the adventitia of the small cerebral arteries resemble closely those already described in the media. The

hyalinization that involves the media spreads also to the adventitia (Fig. 3). Calcification, when it occurs, spreads from within outward, at first involving the portion adjacent to the media but eventually replacing the entire wall (Fig. 4).

In the smallest arteries no differentiation can be made between the middle and outer vessel lamina, since the media early undergoes complete fibrosis and hence merges imperceptibly with the outer lamina.

Basal Vessels: As stated in a previous paragraph, when the small cerebral vessels alone are considered there is very little difference in their structure regardless of the part of the cerebrum considered. There is one possible exception to this statement and that concerns the vessels in the basal nuclei. As a rule these small arteries are thicker walled and contain somewhat more muscle tissue within their medial walls. The small arteries of 50 μ have already lost enough muscle to make them indistinguishable from similar sized vessels elsewhere in the brain.

The age changes already described occur also in the basal vessels, only much more extensively and at an earlier age. Reduplication of the elastica interna can be seen as early as the third decade. The medial musculature disappears very rapidly so that these vessels rapidly assume a fibrous appearance. Hyalinization and calcification of the vessel wall are quite frequent and usually well advanced by the fourth decade. The calcification occurs in the same manner as described for the other cerebral arteries, only it occurs more rapidly and often completely replaces the vessel wall in individuals about 40 years of age.

Discussion

The changes occurring in the cerebral arteries with age have been mentioned in the literature, although in many cases their significance was overlooked. Tuthill,[7] Hackel,[6] and Triepel [1] all described the splitting of the elastica interna with age. Triepel gave a fairly complete description of the elastic reduplication but did not connect this alteration with the age of the patient. Tuthill believed that the abnormalities in the elastica interna were due to the effects of intoxication or infection. Hackel studied the cerebral arteries in 13 cases where the age varied from 15 days to 50 years. He described the splitting of the elastica interna as an old age phenomena.

Rosenblath [8] in studying a series of cases noticed that frequently the small cerebral arteries showed a fibrous tissue increase in their walls. In some vessels this fibrosis was quite extreme and caused the author to suspect that this change might be the cause of various nervous tissue alterations. He believed the fibrosis was caused by some type of chronic inflammation. Binswanger and Schaxel [3] also noticed the fibrous tissue increase in the cerebral arteries of older people. However, they carried their study farther and concluded that the various tissues in the arterial wall possess different growing powers. The collagen retains the greatest growing power and continues to increase after the other elements have ceased. Therefore, in older people the cerebral vessels would show a considerable increase in connective tissue, and a relative absence of elastic and muscle tissue.

It appears from our studies on normal brain tissue that the so-called medial fibrosis is the normal structure for the small cerebral arteries and that this fibrosis merely becomes more extensive with the advance of age. Elastic reduplication, hyalinization and calcification, on the other hand, occur only with advancing age and are not observed in the arteries of young individuals.

Conclusions

1. The average small cerebral artery differs in structure from similar sized vessels elsewhere in the body in that it contains within its media a relative paucity of both elastic and muscle tissue and a predominance of collagenous fibers.

2. The very small cerebral arteries are composed almost strictly of collagenous tissue and may appear as a cerebral fibrosis.

3. With the advance of age the elastica interna of the cerebral arteries becomes reduplicated and frequently loses its normal tinctorial properties. The media undergoes a rapid fibrosis. It frequently shows a hyalinization and more rarely a calcification of all of its elements.

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DESCRIPTION OF PLATE

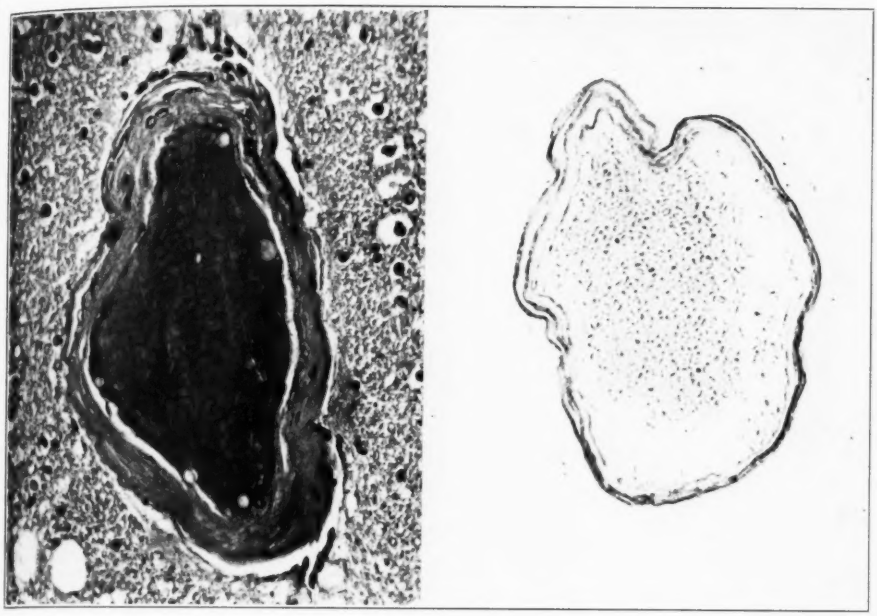
PLATE 80

FIG. 1. Structure of a normal cerebral blood vessel from an individual in the third decade of life. The entire wall is composed of collagenous connective tissue. Only a few cell nuclei are present within the wall elements. Hematoxylin and eosin stain. × 1000.

FIG. 2. Age changes within the elastica interna of a small cerebral artery. Note the reduplication and the patchy and indistinct staining of this membrane. Weigert's elastic tissue stain. × 1000.

FIG. 3. Hyalinization of a cerebral artery. The hyalin is deposited irregularly throughout the vessel wall. Hyalinization is quite extensive in this case and has replaced most of the wall elements. Hematoxylin-eosin stain. × 1000.

FIG. 4. Calcification of a cerebral artery. Note that the deposition of the calcium is limited to the outer portion of the media and the inner layer of the adventitia. The calcium has already formed an almost complete ring within the vessel wall. Hematoxylin-eosin stain. × 1000.



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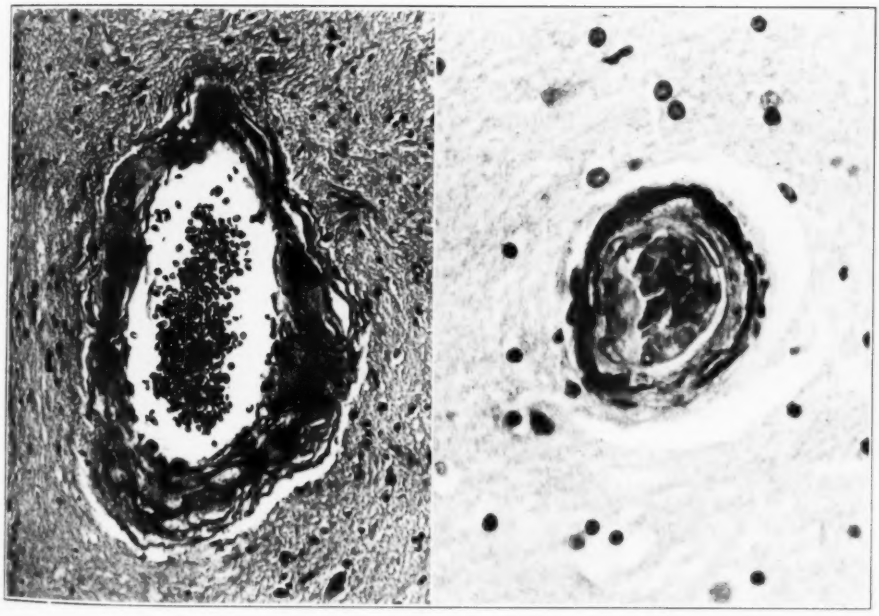

3 4

Baker Structure of Small Cerebral Arteries

UNIV OF MICH

THE HISTOPATHOLOGY OF IDIOPATHIC THROMBOCYTOPENIC PURPURA HEMORRHAGICA *

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INTRODUCTION

Although the literature dealing with the clinical aspects of idiopathic thrombocytopenic purpura hemorrhagica has been voluminous, comparatively little attention has been given to its microscopic pathology. Histological reports of spleens removed surgically have been brief, inconclusive, and have shown a lack of uniformity in the findings. This has led to widely divergent theories often based on insufficient material. Adequate bone marrow studies have been curiously lacking and only a few reports of isolated cases, with examination usually of but one marrow, are to be found. Examination of other organs in autopsied cases has shown no common, significant pathological changes except the fundamental one of widespread hemorrhage.

The finding of strikingly similar histological changes in 2 cases of idiopathic thrombocytopenic purpura hemorrhagica, autopsied by us within a brief period of time, has led to a review of the literature and an investigation of the histopathology of this disease in such material as could be collected.

REVIEW OF LITERATURE

Kaznelson [1] in two spleens removed surgically from cases of idiopathic thrombocytopenic purpura hemorrhagica found numerous megakaryocytes in the "pulp strands" whose presence, he believed, was due to embolic lodgment. The splenic follicles were small and germinal centers absent. Moderate numbers of neutrophilic and eosinophilic polymorphonuclear leukocytes were present in the sinuses. Occasional single platelets were seen in the pulp strands in only 1 case. Unfortunately he was unable to study the bone marrows in these cases.

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Minot [2] examined the bone marrow from a male who died of rapidly fulminating purpura of only 6 weeks duration. The megakaryocytes were plentiful and perhaps increased over normal numbers. He stated that he was unable to tell whether they had any definitely altered histological appearance.

Herzog and Roscher [3] reported 3 cases of secondary thrombocytopenic purpura hemorrhagica. In the 1st case the purpuric manifestations were secondary to massive bone marrow metastases from a primary liver cell carcinoma. The few megakaryocytes seen were apparently normal. The 2nd case (typhoid fever) showed active germinal centers in the splenic corpuscles and rare phagocytosis of platelets in the pulp. The marrow showed suppression of the neutrophilic elements and a lymphoid metaplasia. Megakaryocytes were rare and were thought to show nuclear changes. This case, they believed, showed functional bone marrow damage. The 3rd case, a syphilitic, showed an aplastic marrow, which was qualitatively normal but quantitatively deficient, and megakaryocytes were rare. The spleen had small follicles and showed occasional multinucleated cells in the pulp.

Brill and Rosenthal [4, 5] reported 2 cases of idiopathic thrombocytopenic purpura hemorrhagica. One of the spleens was much enlarged, weighing 1400 gm., and was reported to show hyperplasia of the malpighian bodies and myeloid metaplasia. The second was moderately enlarged and showed only "hypertrophy" microscopically.

Jedlička and Altschuller [6] reviewed 2 cases. Following splenectomy 1 of these cases showed improvement, but in the other case death followed operation. The spleen in the latter case showed, microscopically, inactive germinal centers, many eosinophiles and a few isolated platelets. The bone marrow showed a predominance of neutrophilic myelocytes with very few myeloblasts. There were also many eosinophilic leukocytes and myelocytes. Megakaryocytes were numerous and the majority were reported to show dense structureless nuclei and a ragged, often vacuolated cytoplasm. Granules were irregularly scattered throughout the cytoplasm or gathered into groups. Sternal marrow biopsy of the 1st case showed numerous megakaryocytes with similar, but less marked changes. The spleen apparently was not examined.

Seeliger [7] examined the bone marrow from 2 cases of severe pur-

pura. In each case he studied and compared 200 megakaryocytes, which were present in increased numbers. A great majority contained no azure platelet-forming granules. The cells were poorly defined and vacuolated, and frequently showed degeneration of the nuclei, which contained many nucleoli. The cytoplasm was hyaline and filamented, and contained no cell inclusions. He stated that the findings in the spleen were not characteristic. Megakaryocytes were never found and increased numbers of platelets were seen in only one case.

An autopsied case was presented by Schmincke.[8] The spleen contained megakaryocytes, myelocytes and myeloblasts. Platelets were reported but not in increased numbers. The malpighian corpuscles were small. The femoral marrow showed normal granulopoiesis and erythropoiesis. Megakaryocytes were increased. Most of them were said to show structureless nuclei and often shadow forms. Their cytoplasm was granule-free, irregular and contained many lymphocytes and leukocytes which the author believed to be evidence of phagocytosis. He was tempted to think that megakaryocytes were present in the spleen as compensation for their destruction in the bone marrow, but concluded it was merely myeloid metaplasia.

Two cases were reported by Gaspar.[9] The marrow of 1 case showed increased numbers of megakaryocytes, most of which were interpreted as degenerated forms. There was a smaller proportion of normal appearing young megakaryocytes which, however, were not forming platelets. Only a few platelets were found in the spleen and none of them was phagocytosed. The femoral marrow of the 2nd case showed very few megakaryocytes, all of which were degenerated forms. The spleen contained no platelets.

In a series of collected spleens from cases of essential thrombocytopenic purpura hemorrhagica Gordon [10] found a uniform pathological picture. The follicles were unaffected and the pulp was said to be crowded with proliferating endothelial cells derived from the sinus endothelium. In addition he found phagocytic giant cells which he believed had not been reported previously.

Gerlach [11] reported an autopsied case of Werlhof's disease. The lymph follicles of the spleen were small. He could find no change in the sinus endothelium and found no myeloblasts, myelocytes or eosinophiles. Platelets and megakaryocytes were said to be few. Examination of the liver and lung revealed no megakaryocytes or

platelets. Myeloblasts and neutrophilic myelocytes formed the majority of cells in the femoral marrow. Adult leukocytes of all types were reported absent and there were very few nucleated red blood cells or erythroblasts. There were great numbers of megakaryocytes with large condensed nuclei. They possessed very little cytoplasm, no granules and, to the author, appeared degenerated. Only a few megakaryocytes possessed pseudopods and contained azurophilic granules. No mention was made as to the presence of platelets in the marrow but it was concluded that Wright's [12] theory of platelet formation was correct.

Merklen and Leriche [13] examined a spleen in which the findings are of questionable value because a diagnosis of aleukemic myelosis could not be ruled out.

Twenty-one clinical cases of hemorrhagic thrombocytopenia, with examination of the spleen in 7 cases and three autopsies, were reviewed by McLean, Kreidel and Caffey.[14] The spleens showed no significant changes. Moderate congestion of the sinuses was present in 2 cases and hyperplasia of the malpighian bodies in 1 case. The number of platelets varied from few to large platelet masses. The 3 autopsied cases showed no informative extrasplenic pathological changes except for cerebral hemorrhage in 2 of the cases.

Pemberton,[15] reporting a series of 57 splenectomies for essential thrombocytopenic purpura hemorrhagica, found, on microscopic examination of the spleen, no constant or distinctive pathological changes other than chronic congestion or chronic splenitis. Tuberculosis was present in 2 cases.

Lawrence and Knutti [16] examined the bone marrow in 6 cases of idiopathic thrombocytopenic purpura hemorrhagica. Four cases were clinically of the chronic type. The marrows were normal in 2 of these and megakaryocytes were said to be decreased in the others. The remaining 2 cases, clinically acute, showed hyperplastic marrows with morphological variations in the megakaryocytes.

Sternal biopsies of 4 cases of idiopathic thrombocytopenic purpura hemorrhagica and 1 case of purpura secondary to malignant diphtheria were examined by Willi.[17] In the former cases the megakaryocytes were not pathological in appearance but no platelets were seen. The case of malignant diphtheria showed vacuolization of the cytoplasm of the promegakaryocytes in the marrow. Again no platelets were found.

Krjukof [18] presented a case of Werlhof's disease with spontaneous recovery. Marrow biopsy showed numerous normal appearing megakaryocytes surrounded by many platelets.

Smith [19] reported 1 case successfully treated by splenectomy. Microscopically the spleen showed dilated sinuses with thickened walls. The malpighian bodies were of normal size and the germinal centers often contained degenerated macrophages.

Finally Levrat,[20] who has been the only author to attempt to establish a common pathological picture in spleens from cases of idiopathic thrombocytopenic purpura hemorrhagica, has reported the examination of a spleen from a case of his own and reviewed the descriptions of eleven other spleens reported in the French literature. His case and 5 others showed a hyperplasia of the splenic corpuscles with clear germinal centers in which were many mitoses. Many islands of myeloid tissue were found in the pulp and there was proliferation of the sinus endothelium. He believed these findings to be too common an occurrence to be mere coincidence but could not decide whether they were fundamental to the pathology of purpura or a secondary reaction to consecutive and repeated hemorrhages. The other spleens showed no common findings of significance. One of them contained large macrophages with cytoplasmic granules. This individual later came to autopsy and partial hematopoietic transformation of the lymph nodes was reported. The bone marrow was hyperactive and the megakaryocytes showed morphological alterations which were considered pathological. The spleen from his case showed scattered megakaryocytes which he apparently considered a part of the myeloid metaplasia.

Pathogenesis

Frank [21] believed the disease to be due primarily to a marked platelet reduction through inhibition or destruction, with morphological changes, of the megakaryocytes by some noxa probably arising in the spleen. This theory has the most adherents.[5-9] Minot,[2] agreeing in part with this theory, suggested a specificity of the deleterious agent enabling it to act on the circulating platelets, on the megakaryocytes, or on the marrow as a whole. He stressed the similarity of certain cases of aplastic anemia to those of idiopathic thrombocytopenic purpura hemorrhagica. Kaznelson,[1] however,

believed that the reduction of blood platelets was brought about by an increased thrombolytic activity of the spleen. The increased number of megakaryocytes in the marrow, reported by other authors, he believed was a compensatory hyperplasia. On this hypothesis he introduced splenectomy as a therapeutic measure. In an attempt to explain the purpuric manifestations in cases without thrombocytopenia Glanzmann [22] was the first to postulate a hereditary qualitative deficiency of the platelets.

More recently, with accumulated evidence that thrombocytopenia is not the sole factor in the causation of hemorrhages, the theory has been evolved that in all cases a hyperpermeability of the capillary walls must exist. Brill and Rosenthal [4, 5] concluded that the disease was associated with a disturbed function of the capillary walls, in addition to a disordered state of the blood platelets. They suggested that the changes in the capillary wall might be due to a diminished number of platelets. Payne and Whitehead [23] later came to essentially the same conclusions. It was suggested by Salzman [24] that the spleen may destroy normal numbers of abnormally formed platelets. Krjukof [18] suggested that normally formed platelets may be retained within the capillaries of the bone marrow by a spasm of their walls. He also considered the possibility of some cases falling into the group of allergic manifestations. Combinations of these theories have been suggested by other authors.[16, 25, 26]

Material and Methods

Our material consisted of the following autopsied cases: 5 cases of idiopathic thrombocytopenic purpura hemorrhagica; 3 cases of secondary thrombocytopenic purpura hemorrhagica; 14 cases of symptomatic purpura hemorrhagica; 2 cases of unclassified purpura hemorrhagica; 2 cases of aplastic anemia; 1 case of scurvy; 1 case of hemophilia; 1 case of hemorrhagic disease of the newborn; and 9 normal control bone marrows from cases of sudden death in the various age groups. Also included were spleens removed surgically from 6 cases of idiopathic thrombocytopenic purpura hemorrhagica; 2 cases of unclassified purpura hemorrhagica; and nine spleens from various conditions such as pernicious anemia, hemolytic jaundice, Banti's disease, cirrhosis of the liver, and so on. Of this material, 2 of the cases of idiopathic thrombocytopenic purpura

hemorrhagica, and 1 case of secondary thrombocytopenic purpura hemorrhagica were autopsied by us. The majority of the other material was taken from the autopsy and surgical cases of this laboratory. The remainder was collected from the Peter Bent Brigham Hospital, the Faulkner Hospital, Children's Hospital and from private cases of Dr. A. R. Kimpton.

Tissues from material in this laboratory were fixed in Zenker's fluid, cut at 6 μ and stained with phloxine-methylene blue. The tissues from other sources were fixed in Zenker's fluid, stained with hematoxylin and eosin, and occasionally by Giemsa's method.

Bone marrows were studied by means of differential cell counts after the technique of Krumbhaar and Custer.[27] The optical system employed was a 15 × ocular micrometer of the two parallel line variety, and a 2 mm. oil immersion objective.

Identification and tabulation of 500 cells was done by each author on every marrow and a mean taken. Very little discrepancy was noted between the individual counts. In order to avoid any personal factor a mechanical stage was used and the fields were chosen by moving the stage one division. However, fields containing large fat vacuoles, capillaries and bone trabeculae were avoided, when encountered, by moving the stage one division farther. The number of cells per field varied between 30 and 90 and averaged 60.

Because of the limited number of fields observed in this differential count an accurate index of the number of megakaryocytes present could not be determined, so megakaryocyte counts were made. Following the technique of Krumbhaar and Custer megakaryocytes were counted in 200 consecutive fields, thus giving their percentage in 12,000 cells. Comparison of the individual counts, however, revealed a wide discrepancy. Therefore, megakaryocytes were counted in 400 consecutive fields by each author, a mean taken, and the result expressed as percentage per 24,000 cells. By this technique there was no significant variation.

The megakaryocytes were divided into three types: young, adult and degenerated forms. Those interpreted as young forms were fairly large cells with single, large oval nuclei, occasionally indented and possessing a characteristically arranged, heavy chromatin network. The cytoplasm was scanty, pinkish gray and finely granular. Although pseudopods were not seen the cell outline was occasionally irregular. Adult megakaryocytes possessed a large multilobulated

nucleus with a chromatin network similar to that of the young forms. The cytoplasm was abundant, finely granular and generally projected several pseudopods. In the degenerated form the nucleus was represented by a structureless, dark, compact irregular mass. The cytoplasm varied in amount and was generally free of granules. The cell outline was irregular. It is of interest to note that inclusions of leukocytes and mononuclear cells were found in the cytoplasms of only the late adult and degenerated forms.

Special megakaryocyte stains were done after the technique of Wright [12] and that of Kingsley.[28] However, since these stains require special fixation and comparatively fresh tissue, it was only in three marrows and one spleen that satisfactory results were obtained. Various modifications of the Romanowsky stain (Giemsa, azure II eosin, and Pappenheim's method) were tried. Tissues were fixed in formalin, formalin-Zenker, mercuric chloride and saline, and also in methyl alcohol. With these various techniques no satisfactory results were obtained.

Gross Pathology

Of the 5 cases of idiopathic thrombocytopenic purpura hemorrhagica which were autopsied (35–412 and 35–480; and PBBH 24–116, 25–45 and 35–107) the outstanding gross finding was hemorrhage. There were widespread petechiae and ecchymoses of the skin and mucous membranes in all cases. In 3 cases there was hemorrhage into the serous cavities. In 2 cases in which the head was examined intracranial hemorrhage was found. Other organs involved by hemorrhage were heart, lungs, gastro-intestinal tract pancreas, renal pelves and adrenals. All the spleens were moderately enlarged except one (PBBH 25–45) and all weighed between 95 and 311 gm. The only other constant gross finding of interest was prominence of the malpighian corpuscles. In the 2 cases autopsied by us all the marrows were red and active, except the tibial marrow in 1 case (35–480) which was fatty. Of the other cases it was noticed that the femoral marrow was active in only 1 case (PBBH 25–45).

The 3 autopsied cases of secondary thrombocytopenic purpura hemorrhagica showed similar, widespread hemorrhagic manifestations in 1 case (36–168) and only petechial hemorrhages and ec-

chymoses in the other cases. Splenomegaly was present in only 1 case (PBBH 25–78) and in no case was there any gross abnormality of the spleen. All marrows were active in 1 case (36–168), a child 1 year of age. The femoral marrow was active in another case (PBBH 25–78). The primary lesions in these 3 cases were suppurative otitis media, acute and chronic pancreatitis and pyelonephritis, and hypertensive heart disease.

The 2 autopsied cases of unclassified purpura hemorrhagica are so grouped because of some abnormality of symptomatology, or incompleteness, or discrepancy of laboratory data which makes an exact diagnosis impossible. Both cases presented widespread hemorrhage and the spleens weighed 150 and 979 gm. respectively. The femoral marrow was red and active in 1 case (09–136) but was not examined in the other.

The gross pathological lesions in the cases of scurvy, hemophilia and hemorrhagic disease of the newborn, of the 2 cases of aplastic anemia, and of the 14 cases of symptomatic purpura hemorrhagica, are not of significance in this paper.

All of the six spleens removed surgically from cases of idiopathic thrombocytopenic purpura hemorrhagica were enlarged. Their weight varied from 155 to 390 gm., averaging 227 gm. Grossly they showed prominent malpighian corpuscles but were otherwise negative. The two spleens removed because of an unclassified purpura hemorrhagica were both enlarged. One weighed 300 gm. and the weight of the other was not recorded. The nine spleens removed for other conditions and used for controls showed varying gross lesions which are not pertinent.

Histology of Bone Marrow

The various marrows from the cases of idiopathic thrombocytopenic purpura hemorrhagica were all active and cellular with the exception of one tibial marrow (35–480) which was inactive. The marrows of the unclassified, symptomatic and secondary purpuras were at least normally active. Those of the control cases were considered as normal. The differential cell counts and megakaryocyte counts are given in Tables I to VI.

Differential Marrow Counts

Differential marrow counts could not be done on all available marrows because many were too poorly stained to permit accurate identification of the cells, or there were too few cells in the sections. They were done on the vertebral, femoral and sternal marrows of 2 cases of idiopathic thrombocytopenic purpura hemorrhagica (35–412 and 35–480) and on the rib marrow of 35–480. In 3 cases of symptomatic purpura hemorrhagica the vertebral marrows were counted, and in 1 case of secondary thrombocytopenic purpura hemorrhagica the vertebral and sternal marrows were counted. All vertebral marrows of the 9 control cases were counted. The results of these counts are given in Tables I, II and III.

From these tables it may be concluded that no significant difference exists in the proportion of the various cell elements of the marrows from cases of idiopathic thrombocytopenic purpura hemorrhagica, as compared with those from secondary thrombocytopenic and symptomatic purpura hemorrhagica and normal marrows. There is, however, in the marrows of the first group of cases a slight preponderance of the more mature forms of the erythrocytic series. This is undoubtedly a compensatory phenomenon induced by the prolonged severe hemorrhages. It is to be noted especially that there is no significant change in the percentage of eosinophilic granulocytes. There is very little variation between the percentage composition of the cellular elements of the various marrows of an individual case. Although there is a moderate degree of variation between the percentage composition of cells in the normal marrows of the various age groups they cannot be correlated to form any significant trend. It is interesting to note that in marrow 32–102, a case of severe infection, there was toxic suppression of the granulocyte series, which is brought out very well by the table.

Megakaryocyte Counts

Megakaryocyte counts could not be done on all available marrows because of the same factors encountered in the differential cell counts. They were done on all marrows from cases of idiopathic thrombocytopenic purpura hemorrhagica except 1 (PBBH 25–45) which was considered too fatty. Marrows from 5 cases of symptomatic purpura hemorrhagica were suitable for counting, and two

TABLE I

Idiopathic Thrombocytopenic Purpura Hemorrhagica

Differential Marrow Counts % Based on 500 Cells

Case No.	Sex	Age	Bone marrow	Undifferentiated cells	Granulocyte series									Erythrocyte series				Thrombocyte series				Reticulo-endothelial cells	Lymphocytes	Plasma cells
					Premyelocytes	Eosinophilic premyelocytes	Myelocytes	Eosinophilic myelocytes	Metamyelocytes	Eosinophilic metamyelocytes	Segmented cells	Eosinophilic segmented cells	Total percentage	Stem cells	Erythroblasts	Nucleated reds	Total percentage	Young megakaryocytes	Adult megakaryocytes	Degenerated megakaryocytes	Total percentage			
		yrs.																						
35-412	F	23	Vertebral	2.0	8.6	1.0	20.6	2.6	9.4	0.8	6.0	0.6	49.6	9.2	15.2	22.2	46.6	0.2	0.2	0.0	0.4	1.2	0.0	0.2
35-412	F	23	Femoral	2.0	8.2	0.4	19.2	1.0	14.8	1.2	6.2	0.4	51.4	3.4	13.4	25.4	42.2	0.0	0.8	0.0	0.8	3.2	0.0	0.4
35-412	F	23	Sternal	1.8	4.0	0.2	16.4	3.4	16.8	0.4	7.2	0.4	48.8	4.0	13.4	30.8	48.2	0.0	0.0	0.0	0.0	1.2	0.0	0.0
35-480	M	19	Vertebral	2.7	12.4	0.0	20.7	1.6	13.1	0.8	1.9	0.0	50.5	3.6	10.1	30.4	44.1	0.1	0.6	0.1	0.8	1.7	0.0	0.2
35-480	M	19	Femoral	2.8	4.8	0.6	14.8	1.2	10.0	1.0	2.4	0.4	35.2	4.8	12.2	43.2	60.2	0.4	0.0	0.2	0.6	0.6	0.0	0.6
35-480	M	19	Rib	4.2	10.2	0.2	16.4	0.6	15.2	0.6	1.4	0.2	44.8	9.2	10.6	27.2	47.0	0.0	0.0	0.6	0.6	3.2	0.0	0.2
35-480	M	19	Sternal	2.4	5.0	0.4	13.0	2.0	8.0	0.4	2.6	0.2	31.6	1.8	15.6	47.4	64.8	0.0	0.2	0.2	0.4	0.4	0.0	0.4

TABLE II

Symptomatic and Secondary Purpura Hemorrhagica

Differential Marrow Counts % Based on 500 Cells

Case No.	Sex	Age	Bone marrow	Undifferentiated cells	Granulocyte series									Erythrocyte series				Thrombocyte series				Reticulo-endothelial cells	Lymphocytes	Plasma cells	Cause of death
					Premyelocytes	Eosinophilic premyelocytes	Myelocytes	Eosinophilic myelocytes	Metamyelocytes	Eosinophilic metamyelocytes	Segmented cells	Eosinophilic segmented cells	Total percentage	Stem cells	Erythroblasts	Nucleated reds	Total percentage	Young megakaryocytes	Adult megakaryocytes	Degenerated megakaryocytes	Total percentage				
		yrs.																							
32–102	F	40	Vertebral	2.4	10.8	0.0	19.8	0.4	5.0	0.0	0.4	0.0	36.4	6.4	31.2	17.6	55.2	0.4	0.6	0.4	1.4	3.0	0.0	1.6	Pneumonia, purpura
28–209	F	65	Vertebral	4.4	8.8	0.0	26.2	0.2	10.2	0.4	6.2	0.0	52.0	7.0	20.8	13.2	41.0	0.0	0.2	0.0	0.2	1.8	0.0	0.6	Parotitis, purpura
26–35	F	40	Vertebral	5.0	13.8	0.0	21.8	1.4	17.8	1.2	3.6	0.0	59.6	2.4	19.8	8.2	30.4	0.0	0.6	0.0	0.6	3.4	0.0	1.0	Lobar pneumonia, purpura
36–168	M	1	Sternal	2.4	5.8	0.8	18.0	1.2	12.6	0.8	5.2	1.2	45.6	7.2	12.8	30.4	50.4	0.0	0.4	0.2	0.6	1.0	0.0	0.0	Otitis media, secondary thrombocytopenic purpura
36–168	M	1	Vertebral	2.4	8.8	0.4	22.0	2.0	11.6	0.8	5.4	0.4	51.4	7.6	18.8	17.6	44.0	0.0	0.4	0.2	0.6	1.6	0.0	0.0	Otitis media, secondary thrombocytopenic purpura

TABLE III

Normal Marrow Controls

Differential Marrow Counts % Based on 500 Cells

Case No.	Sex	Age	Bone marrow	Granulocyte series									Erythrocyte series				Thrombocyte series				Reticulo-endothelial cells	Lymphocytes	Plasma cells	Cause of death
				Undifferentiated cells	Premyelocytes	Eosinophilic premyelocytes	Myelocytes	Eosinophilic myelocytes	Metamyelocytes	Eosinophilic metamyelocytes	Segmented cells	Eosinophilic segmented cells	Total percentage	Stem cells	Erythroblasts	Nucleated reds	Total percentage	Young megakaryocytes	Adult megakaryocytes	Degenerated megakaryocytes	Total percentage				
		yrs.																						
34-602	F	34	Vertebral	1.0	8.6	0.6	19.8	5.6	13.6	0.0	7.4	0.0	55.6	8.4	15.2	18.2	41.8	0.0	0.4	0.6	1.0	0.4	0.0	0.2	Pulmonary embolism (20 days post-partum)
35-577	F	24	Vertebral	1.8	8.4	0.8	15.4	4.4	14.2	1.4	8.6	0.4	53.6	10.0	16.0	17.6	43.6	0.2	0.0	0.2	0.4	0.6	0.0	0.0	No anatomical cause (postoperative)
35-481	F	9 mo.	Vertebral	2.4	17.2	1.0	11.8	0.6	10.4	1.4	6.6	1.0	50.0	9.4	21.6	15.2	46.2	0.4	0.2	0.0	0.6	0.8	0.0	0.0	No anatomical cause
35-110	M	33	Vertebral	2.2	9.6	0.6	18.8	1.6	6.0	0.8	1.6	1.0	40.0	7.6	17.0	31.8	56.4	0.2	0.2	0.2	0.6	0.8	0.0	0.0	Syphilitic aortitis, polycystic kidneys
35-284	M	2	Vertebral	1.2	10.0	0.2	21.8	0.0	6.0	0.4	1.2	0.0	39.6	13.4	19.8	24.4	57.6	0.0	0.0	0.4	0.4	0.6	0.0	0.6	Hydrocephalus, pulmonary infarcts
35-343	M	38	Vertebral	2.0	8.8	0.2	17.8	3.2	6.4	0.8	6.6	0.6	44.4	12.4	16.2	22.0	50.6	0.2	0.4	0.0	0.6	2.0	0.0	0.4	No anatomical cause
34-676	M	54	Vertebral	2.6	10.6	0.6	14.4	1.2	12.6	0.4	9.0	0.0	48.8	11.4	16.0	19.2	46.6	0.2	0.4	0.0	0.6	1.2	0.0	0.2	Pulmonary embolism (unhealed fractures of tibia and fibula)
34-467	M	11 mo.	Sternal	1.0	7.2	0.8	14.2	1.2	5.4	0.4	2.4	0.2	31.8	7.6	33.6	24.6	65.8	0.4	0.0	0.0	0.4	1.0	0.0	0.0	Foreign body in bronchus, atelectasis
34-146	F	18	Vertebral	0.8	10.2	0.2	16.4	2.0	7.6	1.8	0.6	0.8	39.6	9.8	14.2	34.6	58.6	0.0	0.2	0.2	0.4	0.6	0.0	0.0	No anatomical cause (postoperative)

marrows of 1 case of secondary thrombocytopenic purpura hemorrhagica were counted. Vertebral marrows on both cases of unclassified purpura were counted as were the vertebral marrows of the 9 normal controls. The results are given in Tables IV, V and VI.

TABLE IV

Megakaryocyte Count
Idiopathic Thrombocytopenic Purpura Hemorrhagica
Percentage per 24,000 Cells Seen in 400 Fields

Case No.	Sex	Age	Bone marrow	Total count		Megakaryocytes		
						Young	Adult	Degenerated forms
		yrs.		*No.*	*%*	*%*	*%*	*%*
35–412	F	23	Vertebral	230	0.920	16	43	41
35–412	F	23	Sternal	109	0.436	21	41	38
35–412	F	23	Femoral	137	0.548	19	53	28
35–480	M	19	Vertebral	72	0.288	17	62	21
35–480	M	19	Sternal	17	0.068	0	12	88
35–480	M	19	Femoral	34	0.136	15	50	35
PBBH 24–116	M	26	Vertebral	94	0.380	8	47	45
PBBH 35–107	F	40	Rib	73	0.292	18	61	21

From a study of the megakaryocyte counts it is seen that the total percentage of megakaryocytes in the normal marrow controls is surprisingly uniform, varying only from 0.24 per cent to 0.423 per cent. In all cases the young forms comprise the smallest group. Adult and degenerated forms appear to alternate almost equally in preponderance.

In the marrows from the cases of idiopathic thrombocytopenic purpura hemorrhagica, however, marked variation is seen, both between different cases and between different marrows of the same case. The vertebral marrow in case 35–412 contains more than twice the number of megakaryocytes present in the highest of the normal controls. The sternal and femoral marrows of the same case show only a slight increase over normal. The count of the vertebral marrow in case 35–480 is in the low normal range, while the sternal and femoral marrows of the same case are definitely below normal. The marrows of the remaining 2 cases are within normal limits.

Both marrows from the case of secondary thrombocytopenic pur-

TABLE V

Megakaryocyte Count

Secondary Thrombocytopenic and Symptomatic Purpura

Percentage per 24,000 Cells Seen in 400 Fields

Case No.	Sex	Age	Bone marrow	Total count		Megakaryocytes			Cause of death
						Young	Adult	Degenerated forms	
		yrs.		*No.*	%	%	%	%	
26–35	F	40	Vertebral	79	0.314	15	43	42	Lobar pneumonia, purpura
28–209	F	65	Vertebral	26	0.102	20	51	29	Parotitis, purpura
32–102	F	40	Vertebral	171	0.682	17	66	17	Pneumonia, purpura
36–168	M	1	Vertebral	146	0.582	20	50	30	Otitis media, secondary thrombocytopenic purpura
36–168	M	1	Sternal	158	0.630	19	48	33	Otitis media, secondary thrombocytopenic purpura
C 28–131	M	11 mo.	Rib	116	0.464	13	40	47	Septic hip, malnutrition, purpura
C 32–158	M	4 mo.	Vertebral	44	0.176	7	59	34	Streptococcus septicemia, otitis media, purpura
									Unclassified Purpura
09–136	F	20	Vertebral	122	0.485	22	54	24	Multiple hemorrhages, bronchopneumonia
PBBH 27-123	M	49	Vertebral	56	0.224	16	29	18	Purpura hemorrhagica

TABLE VI

Megakaryocyte Count

Normal Marrow Controls

Percentage per 24,000 Cells Seen in 400 Fields

Case No.	Sex	Age	Bone marrow	Total count		Megakaryocytes			Cause of death
						Young	Adult	Degenerated forms	
		yrs.		*No.*	*%*	*%*	*%*	*%*	
34-602	F	34	Vertebral	93	0.370	3	22	75	Pulmonary embolism (20 days postpartum)
35-577	F	24	Vertebral	106	0.423	22	45	33	No anatomical cause (postoperative)
35-481	F	9 mo.	Vertebral	61	0.246	21	42	37	No anatomical cause
35-110	M	33	Vertebral	75	0.300	10	33	57	Syphilitic aortitis, polycystic kidneys
35-284	M	2	Vertebral	87	0.348	11	41	48	Hydrocephalus, pulmonary infarcts
35-343	M	38	Vetrebral	99	0.346	17	42	41	No anatomical cause
34-676	M	54	Vertebral	87	0.348	11	63	26	Pulmonary embolism, unhealed fractures of tibia and fibula
34-467	M	11 mo.	Sternal	62	0.254	14	48	38	Foreign body in bronchus, atelectasis
34-146	F	18	Vertebral	76	0.304	17	36	47	No anatomical cause (postoperative)

pura hemorrhagica show moderate elevation of the total megakaryocyte percentage. Of the cases of symptomatic purpura hemorrhagica 2 show moderately increased numbers, 1 a normal number, and 2 a definitely diminished number of megakaryocytes.

Of the 2 cases of unclassified purpura hemorrhagica 1 shows a slight increase and the other an equally slight decrease of the total megakaryocyte count.

Although it is dangerous to generalize from a limited number of cases, from study of the percentage of the three age groups of megakaryocytes in the four groups of purpura hemorrhagica it would seem that with two exceptions a definite trend from normal is evident. There appears to be a distinct increase of young forms, together with a slight increase in adult forms, and a corresponding diminution of the degenerated megakaryocytes.

With the stains employed (phloxine-methylene blue, hematoxylin-eosin and Giemsa) no morphological abnormality was noted in the megakaryocytes observed in these counts. In three marrows (femoral (35–480) and femoral and tibial (36–168)), we were able to stain megakaryocytes specifically by Wright's [12] technique. In both cases the cytoplasm of all the megakaryocytes except the degenerated forms contained abundant azurophilic granules. Many pseudopods were seen and platelets could be seen breaking off from them in normal fashion, after the illustrations of Wright. Many of the actively platelet-forming cells were so small as to make it questionable whether or not they could be identified with routine stains.

Many platelets were seen scattered throughout the marrow, and many were grouped near disintegrating pseudopods. The majority of the platelets were of normal size with, however, a few giant forms being noted.

Histology of the Spleen

On histological examination of the spleens from the 11 cases of idiopathic thrombocytopenic purpura hemorrhagica, fairly constant pathological changes were found. The germinal centers in all cases but 2 were enlarged and active, or hyperactive. The germinal centers in 2 cases only showed areas of necrosis of lymphocytes, fragmentation and phagocytosis of nuclei, occasional infiltration of polymorphonuclear leukocytes and clumps of fibrin (the so-called toxic reaction). In all cases but 2 there was an increase, often marked, in

the numbers of neutrophilic or eosinophilic polymorphonuclear leukocytes, or both, in the sinuses of the pulp.

The most significant finding, in our opinion, was the almost constant presence of megakaryocytes in the sinuses of the pulp. Although in most instances they were comparatively rare, in one spleen they were encountered very frequently and in two others they were surprisingly numerous, as many as four being seen in one high power field. All the cells seen appeared to belong to the young or adult group. With the Kingsley [28] stain, in one spleen (S35–2768 — 35–480) we were able to demonstrate the specific granules in the cytoplasm of these cells. Although pseudopods could be demonstrated, no platelets were positively identified. Whether this was due to staining idiosyncrasy or whether they were actually absent, we are unable to say.

With this finding of megakaryocytes in the sinuses a careful search was made in all spleens for hematopoiesis. In 1 case frequent nucleated red blood cells and myelocytes with occasional mitotic figures were found, and in another case there were several eosinophilic myelocytes and metamyelocytes. However, no stem or early cells of either series were found. Furthermore, the constant location of the megakaryocytes free in the sinuses points to their presence as being an embolic manifestation.

Proliferation of the cells of the sinus endothelium, so frequently mentioned by other authors, was not encountered. When the sinuses are comparatively free of blood, however, the cells of the lining endothelium assume a cuboidal shape, become more prominent, and may simulate hyperplasia.

The above mentioned changes are summarized briefly in Table VII.

The sections of the spleen in 1 of the 4 cases of unclassified purpura hemorrhagica were too faded for accurate interpretation. The remaining 3 all showed enlarged, active germinal centers with toxic reaction present in 2 cases. All showed moderately increased numbers of neutrophilic polymorphonuclear leukocytes in the sinuses, and also an increase in eosinophiles. Rare megakaryocytes were seen in only one spleen. In another scattered areas of erythropoiesis and granulopoiesis were found.

One of the 3 cases of secondary thrombocytopenic purpura hemorrhagica (36–168) was that of severe sepsis. The spleen showed large,

TABLE VII

Data on Spleens from Cases of Idiopathic Thrombocytopenic Purpura Hemorrhagica

Case No.	Weight of spleen	Gross description	Microscopic examination						Other data
			Germinal centers	Toxic reaction	Polymorpho-nuclear leukocytes	Eosinophiles	Megakaryo-cytes	Hema-topoiesis	
	gm.								
S 33–1368	390	Prominent follicles	Occasional large active	None	Many	Many	Occasional	None	
S 35–873	155	Negative	Occasional active	None	Moderate	Moderate	Rare	None	Groups of macrophages laden with fat
S 35–2966	175	Prominent follicles	Large active	None	Moderate	Few	Rare	None	
F 35–367	220	Negative	Large active	None	Rare	Occasional	None	None	Congestion of sinuses
F 34–373	175	Negative	Large active	None	Moderate	Many	Frequent	None	
S 24–92	250	Prominent follicles	Hyperactive	Frequent	Many	Moderate	One	None	
S 35–2768 (35–480)	300	Prominent follicles	Large, often active	None	Many	Few	Many	None	Trabeculae slightly thickened
35–412	220	Prominent follicles	Large active	None	Moderate	Many	Many	None	Congestion of sinuses, edema
PBBH S 24–1002	200	Negative	Occasional large active	None	Many	Few	Occasional	None	Eosinophiles, myelocytes and metamyelocytes
PBBH 25–45	95	Negative	Inactive	None	Few	Rare	Rare		Frequent nucleated reds, many myelocytes with occasional mitotic figures
PBBH S 35–933	311	Prominent follicles	Active	Present	Moderate	Few	Several	None	Many vacuolated macrophages

active germinal centers with a toxic reaction, greatly increased numbers of neutrophilic polymorphonuclear leukocytes in the sinuses of the pulp, and very rare eosinophilic leukocytes. The sinuses contained comparatively little blood, thus making the endothelial lining cells clearly visible and prominent. One megakaryocyte was seen and no hematopoiesis was present. Another case (PBBH 25-78) was that of purpura in a case of long standing hypertension. Here the germinal centers of the malpighian corpuscles of the spleen were inactive and the vessels were hyalinized. Relatively few neutrophilic and eosinophilic polymorphonuclear leukocytes were present, and no megakaryocytes or areas of hematopoiesis could be found. The 3rd case (31-135) in which the purpura was again secondary to infection showed no microscopic abnormalities of the spleen.

In the 14 autopsied cases of symptomatic purpura secondary to various pathological processes sections of spleen were available for examination in all but 1 case. In all cases where the underlying pathology was overwhelming sepsis, notably septicemia and pneumonia, there were greatly increased numbers of neutrophilic polymorphonuclear leukocytes in the sinuses of the pulp. In 3 of these cases occasional megakaryocytes were seen. In all 14 the malpighian corpuscles were small and the germinal centers inactive. In only 2 did they show a toxic reaction. In 1 case eosinophilic leukocytes were prominent. There were clumps of stem cells in one spleen and eosinophilic myelocytes and metamyelocytes in another. Otherwise there was no evidence of hematopoiesis. Another finding in 2 unrelated cases was the occurrence of macrophages filled with phagocytosed red blood cells in the sinuses of the pulp.

In order to evaluate further the microscopic findings in the spleens from cases of idiopathic purpura hemorrhagica nine spleens removed surgically as a therapeutic measure in various pathological conditions were used as controls. Five of these had enlarged malpighian corpuscles with active germinal centers, which in 2 of the cases showed a toxic reaction. Moderate numbers of neutrophilic polymorphonuclear leukocytes were present in 3 cases and were slightly increased in 2 others. In only 1 of these were there more eosinophilic leukocytes than normal. In 3 cases rare megakaryocytes were found. In 1 of these spleens the germinal centers were active and in another there was a slight increase of neutrophilic polymorpho-

nuclear leukocytes in the sinuses of the pulp. In the 3rd case there were a few infarcts. There were no other abnormalities. It is of interest that each of these spleens was removed from a young individual, 10, 25 or 27 years of age, respectively. Also, in each case there was an antecedent history of appendectomy, which in 2 cases was followed by a subphrenic abscess. At the time of splenectomy in each case there were dense adhesions surrounding the liver and spleen, and in 2 cases the liver was noted to be considerably reduced in size at operation. These 3 cases seem to form a clinical group. They all showed megakaryocytes in the splenic sinuses, the significance of which is not clear. Careful search revealed no evidence of hematopoiesis. In two spleens removed from patients with cirrhosis of the liver of another type no megakaryocytes were found.

In a case of Banti's disease there were many large, often multinucleated macrophages containing red blood cells in the sinuses. Moderate hemosiderosis was present in a case of pernicious anemia.

In the spleens from the 2 cases of aplastic anemia that came to autopsy one showed large, active malpighian corpuscles with an occasional toxic reaction. The sinuses contained very few neutrophilic polymorphonuclear leukocytes but numerous phagocytic macrophages. One megakaryocyte was seen. The other case showed occasional masses of organisms in the sinuses with rare leukocytes and no megakaryocytes. The follicles were inactive and the arteriolar walls showed extreme hyalinization.

The spleen from the case of hemophilia showed a toxic reaction of the germinal centers of the malpighian corpuscles. There were numerous plasma cells in the sinuses of the pulp but no evidence of hematopoiesis. Several scattered megakaryocytes were seen. This latter finding is in accord with that of Custer and Krumbhaar,[29] who found megakaryocytes in the splenic sinuses in 2 of their 3 cases of hemophilia.

No sections of spleen were available from the case of scurvy, and the spleen from the case of hemorrhagic disease of the newborn showed no pathological changes of interest.

Histology of Other Organs

The only finding of interest in other organs, apart from hemorrhage, was the occurrence of megakaryocytes in cases of idiopathic

thrombocytopenic purpura hemorrhagica. In 2 of the cases (35–412 and 35–480) they were present in large numbers in the sinuses of the liver and lymph nodes and in the capillaries of the lung. In a 3rd case (PBBH 35–107) the liver only was examined. Several megakaryocytes were seen in the sinuses. This occurrence of megakaryocytes in other organs in addition to the spleen, together with their location always free in blood spaces, excludes any explanation for their presence but that of embolic lodgment. The 2 other autopsied cases of idiopathic thrombocytopenic purpura hemorrhagica showed no megakaryocytes in the liver.

No megakaryocytes were seen in the livers of autopsied cases of unclassified and secondary thrombocytopenic purpura hemorrhagica, symptomatic purpura hemorrhagic, aplastic anemia, hemophilia or scurvy. No evidence of extramedullary hematopoiesis was found in any of the organs examined.

Discussion

From the bone marrow studies it may be inferred that, in certain cases at least, there is no essential difference in the cellular composition of the marrows from cases of idiopathic thrombocytopenic purpura hemorrhagica and other types of purpura hemorrhagica and also from presumably normal marrows.

The megakaryocytes, as seen by routine stains, appear morphologically normal. Specific stains have shown that in 1 case of idiopathic thrombocytopenic purpura hemorrhagica and 1 case of secondary thrombocytopenic purpura hemorrhagica they are apparently functionally normal. This finding is in marked contrast to those of Jedlička and Altschuller,[6] Seeliger,[7] Schmincke,[8] Gaspar,[9] Gerlach,[11] and Willi.[17] Although the latter found morphologically normal megakaryocytes in his 4 cases of idiopathic thrombocytopenic purpura hemorrhagica he stated definitely that there was a temporary complete arrest of platelet formation by the promegakaryocytes, which he considered to be the only platelet-forming type. In our own experience the platelets and megakaryocyte granules have been found to be extremely fragile and very susceptible to destruction by postmortem change and certain methods of fixation. This may possibly partially explain the discrepancy in the findings.

Megakaryocyte counts, compared to those of normal controls,

show increase, decrease or normal numbers. The one constant finding is a preponderance of young and adult forms. This suggests a functional hyperplasia of the megakaryocytes.

We have found a fairly uniform microscopic picture in the spleens of idiopathic thrombocytopenic purpura hemorrhagica. This consists of enlarged, active germinal centers with toxic reaction occurring only rarely. In the majority of the cases there are increased numbers of neutrophilic and eosinophilic polymorphonuclear leukocytes in the splenic sinuses. This increase of eosinophiles is of interest because of the not infrequent slight eosinophilia of the circulating blood and because of the normal numbers of eosinophilic granulocytes in the bone marrows of individuals with idiopathic thrombocytopenic purpura hemorrhagica. The most important, almost constant finding which seems to have been noted but rarely by other authors is the presence of megakaryocytes in the splenic sinuses. This point will be discussed at greater length below.

These findings of enlarged, active germinal centers, increased neutrophilic and eosinophilic polymorphonuclear leukocytes, and the presence of megakaryocytes are met with in other types of purpura and in other pathological conditions, but seldom are they all apparent in the same spleen. We feel that this picture is fairly constant and diagnostic.

Kaznelson [1] found megakaryocytes in two spleens and in the circulating blood of two patients. He interpreted their presence as being due to embolic lodgment and thought this reflected functional hyperplasia of the megakaryocytes in the bone marrow. Our finding of megakaryocytes in the splenic sinuses, in the sinuses of the liver and lymph nodes, and in the capillaries of the lung, in cases of idiopathic thrombocytopenic purpura hemorrhagica, together with the absence of any extramedullary hematopoiesis, would lead us to concur with Kaznelson.

Almost all of the extramedullary megakaryocytes seen were of the young or adult type. Their presence can be explained in only one of two ways. They may represent a maturational arrest of platelet formation, with a throwing out into the blood stream of the functionally active megakaryocytes, or there may be merely a functional hyperplasia of megakaryocytes in the bone marrow and a throwing off of young forms in an effort to compensate for an increased destruction of circulating platelets. This condition would

be analagous to that seen in the erythrocytes in hemolytic anemia. The finding of a slightly increased proportion of young megakaryocytes in the marrow and the demonstration of apparently normal platelet formation favors the latter explanation.

The significance of the finding of varying numbers of megakaryocytes in the marrow is not clear. A possible explanation is that the marrows in which fewer megakaryocytes are found may be showing an early exhaustion of the megakaryocyte series. In this respect it is of interest that the patient whose marrow showed the highest megakaryocyte count came into the hospital in an acute relapse and died in a few hours. The other cases had been hospitalized over a much longer period of time. This offers another explanation for the marrow findings of other authors, in that the megakaryocytes may have been in the end stages of exhaustion. If this be the mechanism the megakaryocyte counts should show a much higher proportion of the degenerated forms. Our counts, however, offer no support to this theory.

Although our findings lead us to believe that the cause of the thrombocytopenia in idiopathic thrombocytopenic purpura hemorrhagica is increased destruction of the platelets rather than inhibition of their formation, we have no evidence as to where this occurs. From clinical evidence, however, the spleen plays an important rôle.

The variation in size and appearance of platelets, so frequently described by other authors, does not necessarily imply that they are functionally deficient. It may also indicate a premature hurried advent into the circulation. Whether or not these platelets are qualitatively defective would seem to be a question for physiological rather than histological investigation.

The theory introduced by Minot [2] that toxins may at times act only on the circulating platelets is strongly supported by our case of secondary thrombocytopenic purpura (36–168). In this case there was a known infection (acute mastoiditis) and a severe thrombocytopenia, but the megakaryocytes in the marrow were increased and actively forming platelets. If this be the mechanism in many cases of purpura secondary to severe infection, the comparatively rare occurrence of megakaryocytes in the blood spaces of the viscera may be explained by the brief duration of this disease as contrasted to idiopathic thrombocytopenic purpura hemorrhagica.

From clinical and laboratory data it is obvious that thrombopenia

is not the sole factor in the causation of hemorrhages. Whether there is a concomitant qualitative defect of the platelets or a capillary hyperpermeability, or both, we are unable to say.

Conclusions and Summary

Differential cell counts have shown no fundamental differences in the bone marrow from cases of idiopathic thrombocytopenic purpura hemorrhagica, various other forms of purpura hemorrhagica and normal controls. Megakaryocyte counts in all forms of purpura show no constant variation except a predominance of the functionally active forms. Under proper technical conditions active platelet formation was demonstrated in the bone marrow from 1 case of idiopathic and 1 case of secondary thrombocytopenic purpura hemorrhagica. A fairly uniform microscopic picture in the spleen and the presence of megakaryocytes in the various organs in cases of idiopathic thrombocytopenic purpura hemorrhagica is reported. The theories of pathogenesis are presented and evidence is introduced that thrombocytolysis is the cause of the thrombocytopenia. It is suggested that this in turn causes a compensatory hyperplasia of the megakaryocytes in the bone marrow. The literature on the histopathology of idiopathic thrombocytopenic purpura hemorrhagica is briefly reviewed.

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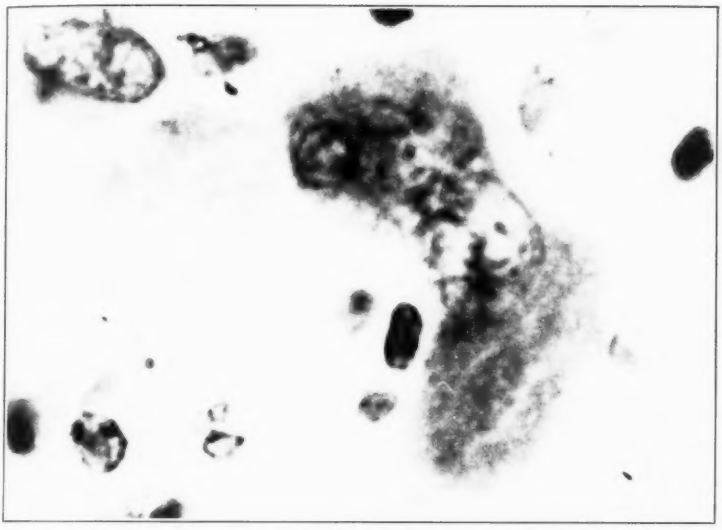
DESCRIPTION OF PLATES

PLATE 81

FIG. 1. Adult megakaryocyte showing normal numbers of granules in cytoplasm. From the femoral marrow of a case of idiopathic thrombocytopenic purpura hemorrhagica (35–480). Wright's megakaryocyte stain. × 1425.

FIG. 2. Spleen from same case (35–480) showing three adult megakaryocytes in sinuses. Phloxine-methylene blue stain. × 450.



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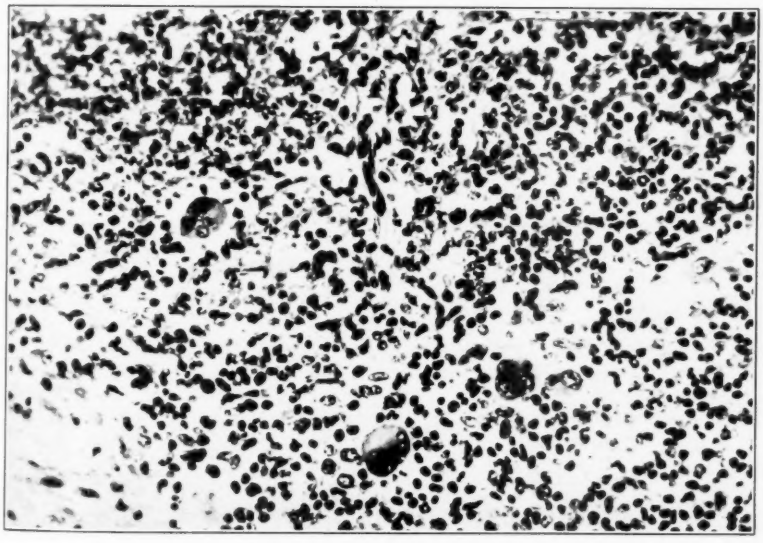

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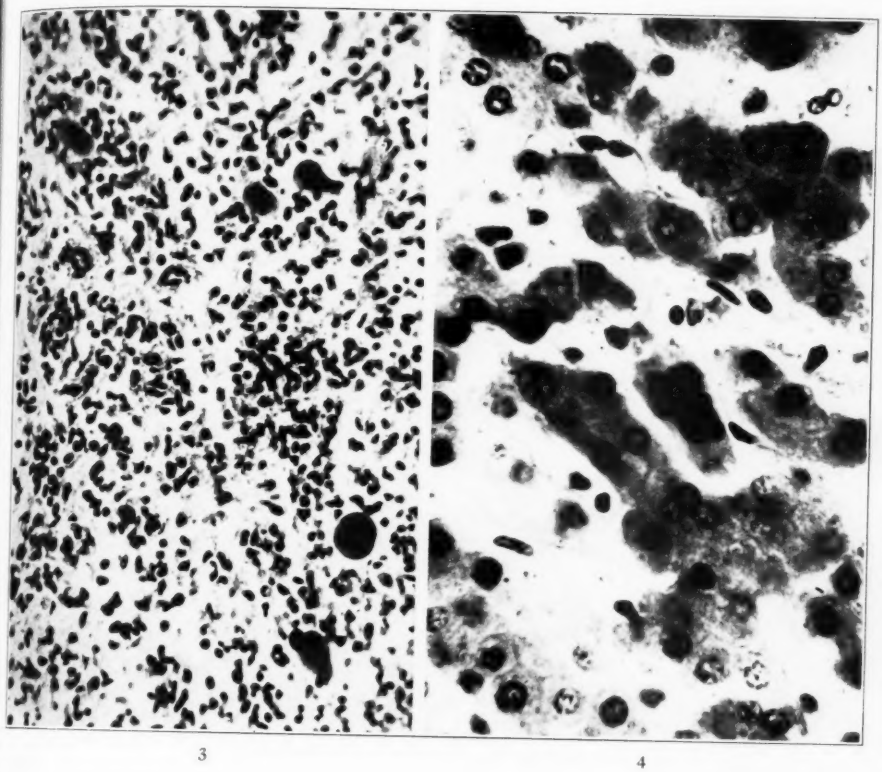
PLATE 82

FIG. 3. Spleen from another case of idiopathic thrombocytopenic purpura hemorrhagica (35–412) showing five megakaryocytes in sinuses. The only degenerated form shows beginning inclusion of a leukocyte. Phloxine-methylene blue stain. × 450.

FIG. 4. Liver (35–480) showing an adult megakaryocyte in sinusoid. Phloxine-methylene blue stain. × 930.

FIG. 5. Retroaortic lymph node (35–412) showing two megakaryocytes in sinuses. Phloxine-methylene blue stain. × 930.



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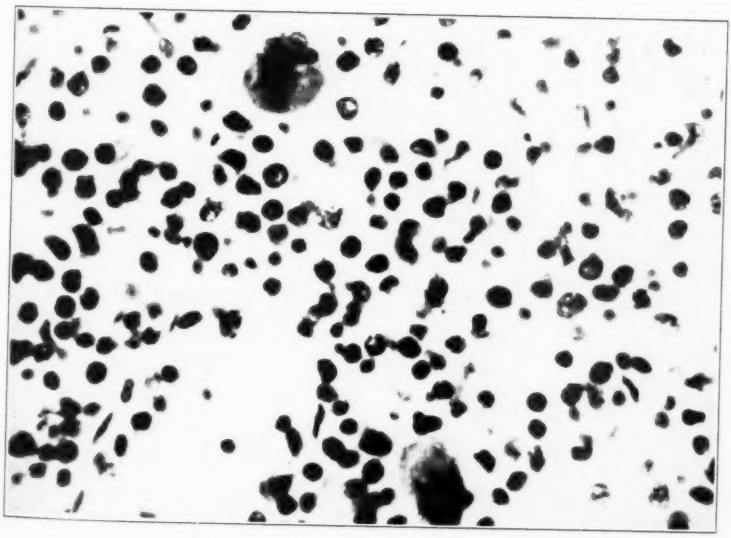

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A NEW METHOD FOR THE RAPID STAINING OF MYELIN SHEATHS *

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The various methods that have so far been devised for staining the myelin sheaths of nerve fibers suffer from one of two disadvantages, either an undue complexity of the method or too great a length of time necessary for its execution. Weigert's methods, [1,2] or any of the several modifications such as those of Pal [3] and Kultschitzky, [4,5] necessitate long periods of mordanting the entire block of tissue before embedding in a solution of either copper or chromium salts, or both. Preparation of the sections by these methods is so time consuming that a period of weeks or months must elapse before they are ready for study. Furthermore, these methods not only do not stain the cells, but the process of mordanting the blocks of tissue so alters the cellular elements that poor results are obtained when an attempt is made to stain the cells by other methods.

The iron hematoxylin method devised by Heidenhain [6] for staining tissues in general has been adapted to the staining of myelin sheaths by several investigators including Loyez,[7] Spielmeyer,[8] Morgan,[9] Weil,[10] and Clark and Ward.[11] While all of these procedures require much less time than Weigert's original method, or its modifications, none of them combines the qualities of reliability, simplicity and rapidity to such a degree as the method described in this report.

The method described herein may be performed in from $\frac{1}{2}$ to 1 hour, depending on the thickness of the sections. It is applicable to normal or pathological material and works well on frozen, paraffin or celloidin sections. It has the further advantage that only the sections to be stained need be mordanted so that sections adjacent to the myelin stained ones may be stained by other methods. In addition, the chromophilic substance may be stained with excellent results in the section that has been stained for myelin sheaths. The myelin is stained a deep blue and the background remains either colorless or

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slightly tinted, depending on the degree of differentiation. The nuclei of all the cellular elements are stained in such a manner that the nuclear structure is clearly shown and it is therefore possible to differentiate the various types of glial cells from each other and from the nerve cells. The chromophilic substance may be stained in the same section by the use of neutral red, thionin or a similar stain.

Directions

1. Mordant sections for 15 minutes in a 4 per cent aqueous solution of ferric ammonium sulphate ($FeNH_4(SO_4)_2$) prepared from violet crystals.
2. Rinse in 70 per cent alcohol to remove excess of mordant.
3. Place sections in a solution of 1 per cent hematoxylin containing 2 to 3 per cent glacial acetic acid by volume. (The 1 per cent hematoxylin solution is made by the addition of distilled water to a 10 per cent stock solution of hematoxylin in absolute alcohol.) The solution containing the sections is kept at or near 55° C. The sections at first stain deeply, then differentiate, and the depth of staining of the myelin sheaths depends on the time the sections are left in the staining-differentiating solution. Usually between 30 and 60 minutes are required but thin sections may require less time.
4. Place in a half saturated solution of lithium carbonate for 5 to 10 minutes.
5. Rinse in tap water.
6. Counterstain or stain for chromophilic substance if desired.
7. Dehydrate in alcohol, clear in xylol and mount in balsam.

Comment

This method gives excellent results with paraffin, celloidin or frozen sections after a number of fixatives, including formalin, alcohol-formalin, and Bouin's fluid. Sections of brain stem which had been preserved in formalin for 5 years stained well. Fixatives containing copper or chromium salts, *e.g.* Zenker's fluid, are not applicable to this method since differentiation will not occur after their use.

Frozen sections can be cut directly from the fixative and stored either in 10 per cent formalin or in 70 per cent alcohol, from which they may be taken and placed directly in the mordant. Paraffin

sections can be stained mounted or unmounted, as desired, but it is necessary to remove the paraffin before the method is applied. In the case of celloidin sections the celloidin need not be removed for no stain is left in it at the completion of the process. On the other hand, if it is so desired, the celloidin may be removed without adverse effects, either before or after the staining process is completed.

The 4 per cent ferric ammonium sulphate mordant should be made from violet crystals and the solution can be used repeatedly as long as it remains clear and without a precipitate. Sections may be placed in the mordant directly from 70 per cent or 80 per cent alcohol, in which they have been stored, or from 10 per cent formalin solution or water. A period of 15 minutes in the mordant is ample; a longer time does no harm and, when convenient, a group of sections may be mordanted overnight and the process completed the next day. Sections that have been mordanted for 2 weeks have been successfully stained. After mordanting the sections are rinsed in 70 per cent alcohol in order to remove the excess of the ferric ammonium sulphate solution. Alcohol is used in preference to water because of the tendency of the water to remove too much of the mordant from the tissue.

The staining solution used in this procedure is unique in that the sections not only stain but also differentiate in the same solution. The staining-differentiating solution is prepared from a 10 per cent stock solution of hematoxylin in absolute alcohol. This solution does not require ripening and it may be used immediately after preparation. Ripening, on the other hand, does not affect the quality of the results. In order to avoid overripening about a month's supply is usually made up in advance and kept tightly corked. Just before using, the required amount of the 10 per cent stock solution is diluted to 1 per cent by the addition of distilled water and enough glacial acetic acid is added so that the solution contains 2 to 3 per cent glacial acetic acid by volume. This gives a sufficiently rapid differentiation in nearly all instances, but if a more rapid differentiation is desired the solution may be made up to contain as much as 8 per cent glacial acetic acid without any apparent harm to the sections. In every instance it is important that the sections be observed frequently in order to prevent differentiation from proceeding too far. A convenient way to handle the sections is to place them in the stain in Petri dishes on a slide warming table. Care

should be taken to make certain that the sections are entirely covered by the staining-differentiating solution and do not lie upon one another, otherwise an irregular differentiation occurs. When the section has been differentiated to the desired degree it is placed in a half saturated solution of lithium carbonate for about 5 minutes. In the lithium carbonate solution not only is the differentiation stopped but the hematoxylin that has not been fixed in the tissues diffuses into the solution. The section is then placed in tap water for a few minutes, dehydrated in graded alcohols and mounted in neutral balsam. If it is necessary to keep stained sections a day or two before mounting they should be placed in tap water since storage in alcohol causes the stain to fade slowly.

Occasionally it may be found necessary to interrupt the process of differentiation before it has been completed. Under such circumstances the section may be taken from the staining-differentiating solution, placed for 5 minutes in a half saturated solution of lithium carbonate and then transferred to tap water where it may be kept for as long as several days. When convenient the differentiation may be completed by placing the section in the staining-differentiating solution at 55° C. If for any reason the differentiation has been allowed to proceed too far, the section may again be mordanted and the staining-differentiating process repeated.

The advantage this method offers in making possible the staining of sections for myelin sheaths and the adjacent sections for other structures is further enhanced by the fact that a cell stain may be used on the myelin stained sections. Although the cytoplasm of the nerve cells is tinted and the chromophilic granules are usually faintly discernible in the sections, they are not stained well enough for careful study. If, therefore, it is desired to study the chromophilic substance in the myelin stained sections they can be stained in the usual manner by any of the chromophilic stains such as thionin, toluidine blue or neutral red. We have found that a 1 per cent aqueous solution of neutral red not only stains the chromophilic granules well but also affords a good contrast stain.

In addition to staining the myelin sheaths this procedure affords a nuclear stain of such excellence that details of nuclear structure are clearly shown and differentiation of cell types is possible on this basis. All of the cells show a more or less distinct nuclear membrane. The large nucleus of the nerve cell possesses a conspicuous black

staining nucleolus and an extremely delicate, net-like arrangement of nuclear chromatin. The nucleus of the astrocyte, usually about the size of that seen in the small nerve cells, possesses no such large and conspicuous nucleolus but usually contains a number of small deeply stained granules, two or three of which are decidedly larger than the others. The nuclei of the oligodendroglia appear smaller than either of the preceding and contain several relatively large, deeply staining, irregularly shaped chromatin masses scattered throughout the nucleoplasm. The microglia possess the smallest and most elongated nuclei of all the glial cells and have many deeply stained chromatin granules within the nucleoplasm. Red blood cells stain black, a characteristic that renders areas of vascular congestion or extravasation easily recognizable microscopically. The nuclear structure of endothelium, smooth muscle and connective tissue is clearly shown.

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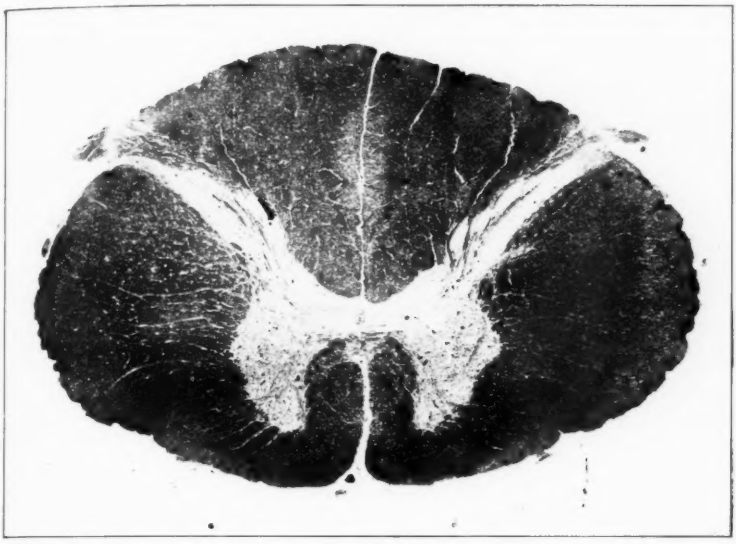
DESCRIPTION OF PLATES

PLATE 83

FIG. 1. Human spinal cord stained by the new method showing the sharp demarcation between the white and gray matter and the amyelinic zone of the entering dorsal root. Formalin fixation, celloidin section, 20 μ. × 10.

FIG. 2. A portion of the dorsal columns from Fig. 1 showing the definitive staining of the myelin sheaths. Formalin fixation, celloidin section, 20 μ. × 500.

FIGS. 3, 4, 5 and 6. Nuclei of astrocyte, oligodendroglia, microglia and nerve cell from a myelin stained section of the cerebral cortex of the dog. Formalin fixation, celloidin section, 20 μ. × 1500.



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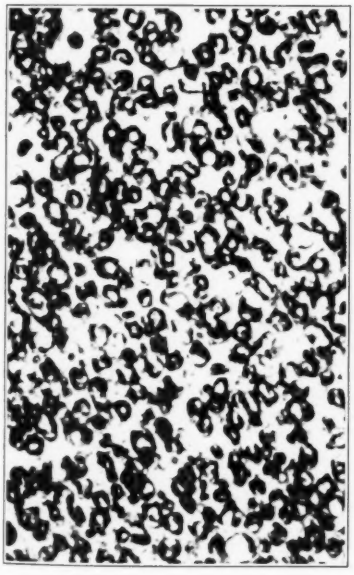

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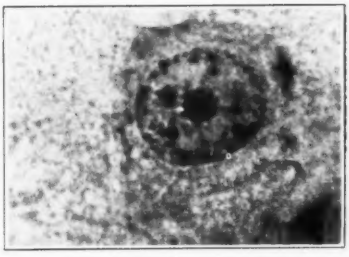

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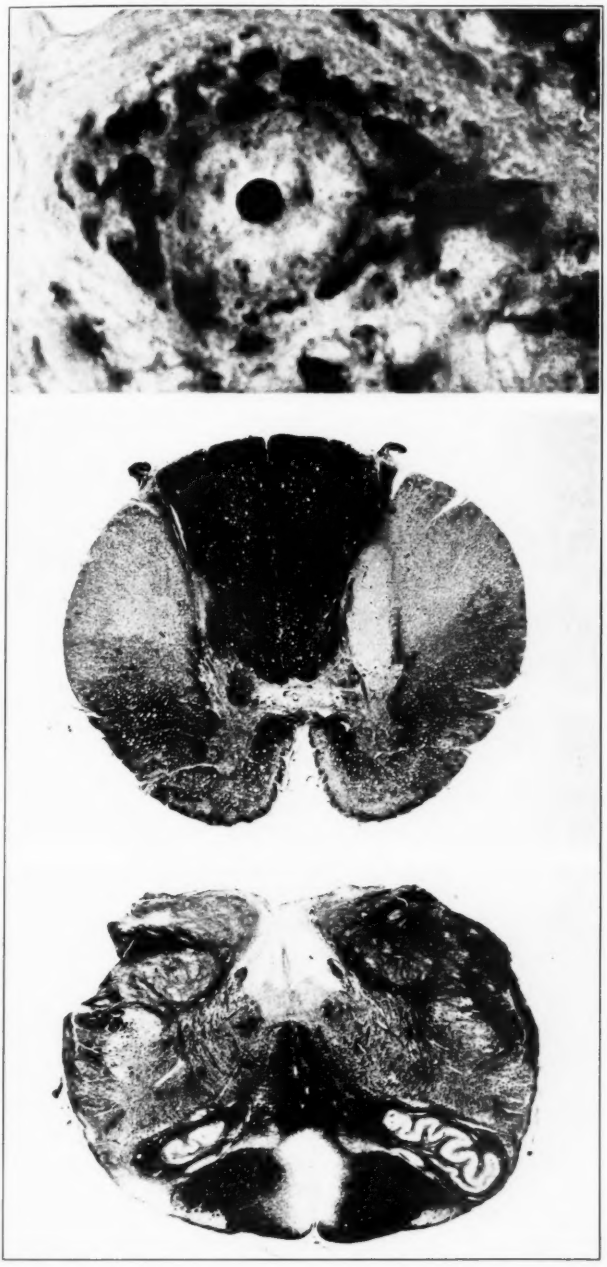
PLATE 84

FIG. 7. Nerve cell from a section of normal spinal cord stained with neutral red after the myelin sheath stain. The chromophilic granules have retained their normal arrangement and ability to stain. Formalin fixation, celloidin section, 20 μ. × 1500.

FIG. 8. Spinal cord from a case of myelitis. Myelin sheath stain. Formalin fixation, paraffin section, 20 μ. × 10.

FIG. 9. Section through the medulla oblongata from a case of multiple sclerosis. The demyelinized plaques are clearly defined by this staining method. Formalin fixation, celloidin section, 20 μ. × 5.



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